Maternal Control of Nodal Signaling: Regulation of Squint Translation

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Maternal Control of Nodal Signaling: Regulation of Squint Translation

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

KUMARI POOJA

22 August 2013

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TABLE OF CONTENTS

Declaration

Acknowledgements ii

Table of Contents iv

Summary x

List of Figures xii

List of Tables xiv

Abbreviations xv

List of Publications xviii

1. Chapter 1 – Introduction 1

1.1 Maternal control of embryonic development 2

1.1.1 Embryonic polarity, cell fate specification and axial patterning 2

1.1.2 Germline Specification 6

1.1.3 Genomic imprinting and chromatin remodeling 8

1.1.4 Maternal to zygotic transition (MZT) 9

1.2 Regulation of maternal RNAs 12

1.2.1 RNA localization 12

1.2.2 Cellular machinery for RNA localization 15

1.2.3 Translational repression and RNA storage 20

1.2.4 Transport and translation are linked 22

1.2.5 Translational activation of Maternal RNAs 25

1.3 Zebrafish as a model organism 26

1.3.1 Maternal effect studies 27

1.3.2 Zebrafish development and dorsal specification 29

1.3.3 Role of maternal *squint* in dorsal specification 30

1.4 Research objectives 33

2. Chapter 2 – Materials and Methods 35

2.1 Molecular Biology and Recombinant DNA techniques 36

2.1.1 Generation of Constructs 36

2.1.2 Semi-quantitative and Quantitative RT-PCR 37

2.1.3 Capped mRNA and DIG labeled antisense probe synthesis 38

2.2 Maintenance of Zebrafish and embryo manipulation 39

2.2.1 Zebrafish Strains 39

2.2.2 Generation of *ybx1* mutant zebrafish 39

2.2.3 Generation of *ybx1* rescue transgene 40

2.2.4 Genotyping mutants 40

2.2.5 Temperature shift experiments 44

2.2.6 Fluorescent capped RNA and morpholino injections 44

2.1.7 Lefty RNA injections 44

2.2.8 Bead Implantation 45

2.3 Biochemistry 45

2.3.1 RNA gel-shifts and UV-crosslinking assays 45

2.3.2 Chromatographic purification of proteins 46

2.3.3 RNA immunoprecipitation 47

2.3.4 Protein expression and detection 47

2.4 Staining and Imaging techniques 48

2.4.1 RNA in-situ hybridization 48

2.4.2 Membrane and nuclear staining 50

2.4.3 Microscopy 51

3. Chapter 3 – Results 55

3.1 Mapping of sqt Dorsal Localization Element (DLE) 56

3.1.1 sqt DLE consists of both sequence and structure 59

3.1.2 ATG morpholinos against sqt 5'UTR also block localization 61

3.2 Identification and purification of DLE binding factors 64

3.2.1 Several factors bind to sqt 3'UTR 64

3.2.2 SRBF1 specifically binds to sqt DLE 64

3.2.3 Purification of sqt RNA binding factor 1 (SRBF1) 67

3.2.4 Purification of sqt RNA binding factor 3 (SRBF3) 70

3.2.5 SRBF1 is the nucleic acid binding protein Y box-binding protein1 (Ybx1) 73

3.2.6 Ybx1 forms protein-RNA complex *in vivo* with sqt RNA 75

3.2.7 Recombinant Ybx1 binds sqt DLE but nor wnt8a or vg1 3' UTRs 75

3.2.8 Ybx1 binding to DLE requires additional elements in sqt coding sequence 75

3.2.9 The N-terminus of Ybx1 is required for binding sqt RNA 78

3.3 Maternal Ybx1 is essential for early development 81

3.3.1 *ybx1* RNA and protein is not spatially restricted 81

3.3.2 *ybx1* mutant alleles 81

3.3.3 M*ybx1* mutant embryos fail to initiate gastrulation movements 85

3.3.4 Maternally expressed *ybx1* transgene rescues gastrulation defects in mutants 87

3.4 sqt RNA localization is disrupted in M*ybx1* embryos 89

3.4.1 sqt RNA fails to localize to future dorsal in M*ybx1* embryos 89

3.4.2 Maternal *ybx1* transgene rescues sqt RNA localization in mutants 89

3.4.3 Localization of other transcripts is not affected in M*ybx1* embryos 91

3.5 sqt RNA processing and Sqt translation is precocious in M*ybx1* embryos 93

3.5.1 sqt RNA levels are marginally reduced in M*ybx1* embryos 93

3.5.2 Polyadenylation and splicing events are accelerated in M*ybx1* embryos 93

3.5.3 Sqt protein translation is premature in M*ybx1* embryos 95

3.5.4 Ybx1 interacts with 5' m7G cap binding protein eIF4E 97

3.6 Nodal signaling is elevated in M*ybx1* embryos 99

3.6.1 Phosphorylation of Smad2 is precocious in M*ybx1* embryos 99

3.6.2 Expression of target genes of Nodal signaling is elevated in mutants 99

3.6.3 Expression of YSL genes is also elevated in mutants 101

3.7 The extra-embryonic YSL is expanded in M*ybx1* embryos 101

3.7.1 Nuclear and membrane staining shows expanded YSL in mutants 101

3.7.2 M*ybx1* embryos show early YSL formation 102

3.8 Nodal diffusion from yolk leads to expanded YSL 105

3.8.1 Wild type embryos implanted with Nodal beads show more YSL nuclei 105

3.8.2 Nodal bead implantation in MZ*oep* embryos does not lead to more YSN 105

3.9 Blocking Sqt/Nodal Signaling can rescue gastrulation arrest in M*ybx1* embryos 107

3.9.1 Overexpression of Lft1 can rescue gastrulation arrest in Mybx1 embryos 107

3.9.2 YSL and gastrulation defects in M*ybx1* mutant embryos are rescued in M*ybx1;sqt* compound mutants 109

4. Chapter 4 – Discussion 111

4.1 Post transcriptional regulation of maternal sqt RNA 112

4.2 Biochemical purification of DLE binding factor, Ybx1 114

4.3 Ybx1 – A multifunctional protein 115

4.4 Specificity of sqt-Ybx1 interaction 116

4.4.1 Modular design of RNA binding proteins confer specificity 116

4.4.2 Components of the ternary complex provide specificity 117

4.5 Conditional disruption of Ybx1 118

4.6 Functions of Ybx1 in sqt RNA localization, processing and translation 118

4.7 Sqt/Nodal signaling and YSL expansion 121

4.8 Evolutionary conserved role of Ybx1 binding to sqt 3'UTR 123

4.9 Translational control of nodal signaling: implications in disease and stem cell pluripotency 125

5 References 126

Summary

We are interested in studying the earliest events during embryonic patterning and axis formation. Since gene expression is minimal during early embryonic stages, before the zygotic transcription, maternal RNAs and proteins deposited during oogenesis play a major role in the earliest events of embryonic patterning. We use zebrafish as a model to study roles of maternal factors during vertebrate development. Maternal mRNA encoding the Nodal-related factor, Squint (Sqt), is asymmetrically localized to 2 cells by the 4-cell stage, and predicts embryonic dorsal. More recently we showed that dorsal axis formation by maternal squint is mediated by a non-coding function of the RNA. The sqt 3' untranslated region (UTR) is both necessary and sufficient for dorsal localization and we have mapped the dorsal localization element (DLE) to first 50 nucleotides of the sqt 3' UTR.

In my PhD thesis work, I analyzed the sqt RNA localization machinery and roles of localized sqt RNA and Sqt/Nodal signaling in embryonic patterning. Nodal signaling is critical for embryonic germ layer patterning, axis formation, and maintenance of human embryonic stem cell pluripotency. Precise and timely regulation of Nodal signaling is also critical since deregulated signaling is associated with metastasizing tumors. We found that maternal Y box-binding protein 1 (Ybx1) is required for asymmetric localization of sqt RNA and translational control by Ybx1 regulates Nodal signaling. Ybx1 was purified and identified as an RNA-binding factor that binds the dorsal localization element (DLE) in the 3' UTR of nodal related-1/squint RNA (sqt). Ybx1 belongs to the cold-shock domain family of conserved multifunctional proteins that regulate gene expression at the transcriptional and translational levels. The N-terminus of Ybx1 is required for sqt RNA-binding, and we have identified the key residues that mediate this interaction. Using ENU-induced and zinc finger nuclease-mediated mutations affecting the *ybx1* locus, we found that loss of maternal Ybx1 function leads to gastrulation failure and embryonic lethality. These phenotypes can be rescued by maternal *ybx1* transgenes. Consistent with binding of Ybx1 to the sqt DLE, I found that localization of maternal sqt RNA is disrupted in *ybx1*

mutant embryos. Interestingly, sqt RNA processing and translation is precocious in *ybx1* mutant embryos. Remarkably, Squint/Nodal target genes are prematurely expressed in mutant embryos, indicating precocious and unregulated Nodal signaling. Consequently, mutant embryos show precocious extra-embryonic yolk syncytial layer (YSL) formation, and fail to initiate gastrulation. Implantation of Nodal-coated beads into the yolk of WT blastula stage embryos can phenocopy *ybx1* mutant defects and blocking Nodal signaling can rescue the gastrulation arrest.

Taken together my results suggest that maternal Ybx1 prevents ectopic Nodal signaling by translational inhibition, and reveal a new paradigm in regulation of Nodal signaling, which is likely to be conserved. These findings also highlight the role of maternal factors in the control of early embryonic development and cell fate specification in vertebrates.

List of Figures

Figure 1.1	Establishment of embryonic axis	5
Figure 1.2	Models of germ cell specification	7
Figure 1.3	Maternal to Zygotic transition (MZT) in various model organisms	10
Figure 1.4	Regulation of maternal RNAs	13
Figure 1.5	Mechanisms underlying mRNA localization	16
Figure 1.6	Translational repression	23
Figure 1.7	Maternal effect genes affecting zebrafish development at various stages	28
Figure 1.8	Schematic outline of Nodal signaling pathway	32
Figure 1.9	Schematic elucidating role of maternal squint transcripts and related questions	34
Figure 2.1	Genotyping strategies	42
Figure 3.1	Deletion analysis identifies sqt Dorsal Localization Element (DLE)	57
Figure 3.2	Mutagenesis defines localization motifs	60
Figure 3.3	Morpholinos targeting the sqt ATG and DLE regions disrupt sqt RNA localization.	62
Figure 3.4	sqt 3'UTR is rich in RNA binding sites	65
Figure 3.5	SRBF1 specifically binds to sqt DLE	66
Figure 3.6	Purification of SRBF1	68
Figure 3.7	Purification of SRBF3	71
Figure 3.8	Alignment of Ybx1 sequences from different species	74

Figure 3.9	SRBF1 is the conserved nucleic acid binding protein Ybx1	76
Figure 3.10	Ybx1 binding requires elements in sqt coding sequence.	77
Figure 3.11	Ybx1 binds sqt DLE via its RNA binding domain	79
Figure 3.12	Expression of ybx1RNA and Ybx1 protein in wild-type embryos	82
Figure 3.13	Ybx1genetics mutants	84
Figure 3.14	Maternal Ybx1 is essential for embryogenesis	86
Figure 3.15	Maternal *ybx1* transgene rescues gastrulation arrest in M*ybx1* mutant embryos	88
Figure 3.16	sqt RNA localization is disrupted in M*ybx1* mutant embryos.	90
Figure 3.17	Localization of other RNAs is not affected in M*ybx1* mutant embryos	92
Figure 3.18	sqt RNA is processed prematurely in M*ybx1* mutant embryos	94
Figure 3.19	sqt RNA is precociously translated in M*ybx1* mutant embryos	96
Figure 3.20	Ybx1 interacts with 5’ m7G CAP binding protein eIF4E	98
Figure 3.21	Nodal signaling is deregulated in M*ybx1* mutant embryos	100
Figure 3.22	M*ybx1* mutant embryos have expanded extra-embryonic YSL	103
Figure 3.23	Nodal bead implantation in the yolk results in more YSL nuclei	106
Figure 3.24	Lefty1 overexpression restores YSL expansion and gastrulation defects in M*ybx1* mutant embryos	108
Figure 3.25	YSL and gastrulation defects in M*ybx1* embryos are rescued by blocking Nodal signaling	110
Figure 4.1	Graphical Summary	122
Figure 4.2	Ybx1 binding to nodal 3’UTR is conserved	124

List of Tables

Table 2.1	Primer Sequences	52
Table 2.2	Morpholino Sequences	54
Table 3.1	Mutations identified in ybx1 locus by zinc finger nuclease injection	83

Abbreviations

4EBP	eIF4E binding protein
4EHP	eIF4E homology protein
A/P	anterior posterior
ATP	adenosine triphosphate
A/V	animal vegetal
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cDNA	complementary deoxy ribo nucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding
CSD	cold shock domain
CTP	cytosine triphosphate
DAPI	4',6-Diamidino-2-phenylindole
DD	dimerization domain
DEPC	diethyl polycarbonate
DIG	digoxygenin
DLE	dorsal localization element
DMSO	dimethyl sulphoxide
DNA	deoxy ribo nucleic acid
DTT	dithiothreitol
D/V	dorsal ventral
EDTA	ethylene diamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
ENU	N-ethyl-N-nitrosourea
EVL	enveloping layer
FGF	fibroblast growth factor

GFP	green fluorescent protein
GTP	guanosine triphosphate
HRP	horse radish peroxidase
IgG	Immunoglobulin G
IPTG	isopropyl β-D-1-thiogalactopyranoside
LE	localization element
m7G	7-methyl guanosine
MAB	maleic acid buffer
MBT	mid blastula transition
MO	morpholino
mRNA	messenger ribo nucleic acid
mRNP	messenger ribo nucleo protein
MZT	maternal to zygotic transition
MZ	maternal zygotic
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NTMT	alkaline phosphatase buffer
PABP	polyadenylation element binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cell
qPCR	quantitative real time PCR
RBP	RNA binding protein
RIPA	radio immuno precipitation assay
RNA	ribo nucleic acid
RNA IP	RNA immunoprecipitation
RT PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SRBF	sqt RNA binding protein

SSC	saline sodium citrate
ssDBD	single stranded DNA binding domain
TBE	tris/Borate/EDTA
Tg	transgene
TILLING	Targeting Induced Local Lesions in Genomes
UTP	uracil triphosphate
UTR	untranslated region
WT	wild type
YSL	yolk syncytial layer
YSN	yolk syncytial layer nuclei
ZFN	zinc finger nuclease
ZGA	zygotic genome activation

List of Publications

Pooja Kumari, Patrick C. Gilligan, Shimin Lim, Tran Duc Long, Sylke Winkler, Robin Philp, Karuna Sampath. An essential role for maternal control of nodal signaling. *eLife*, 2013; 2:e00683.

Shimin Lim, **Pooja Kumari**, Patrick Gilligan, Helen Ngoc Bao Quach, Sinnakaruppan Mathavan and Karuna Sampath. Dorsal Activity of maternal squint is mediated by a non-coding function of the RNA. *Development*, 2012 139:2903-2915.

Patrick C. Gilligan, **Pooja Kumari**, Shimin Lim, Albert Cheong, Alex Chang and Karuna Sampath. Conservation defines functional motifs in the squint/nodal-related 1 RNA dorsal localization element. *Nucleic Acids Research*, 2011 39:3340-3349.

Chapter 1

Introduction

1.1 Maternal control of embryonic development

In animals, development starts with the formation of gametes in the adult gonads and embryogenesis begins after the event of fertilization when haploid gametes fuse to give rise to a diploid zygote. Upon fertilization, the sperm nucleus enters the oocyte and triggers the developmental program which originally started during oogenesis. An oocyte is a specialized cell capable of regulating multiple cellular and developmental processes. The maturation of an oocyte is a complex molecular process during which it accumulates all the components required for completion of meiosis, fusion of two haploid genomes, initiation of mitosis, early embryo metabolism, as well as activation of zygotic transcription at the right time (Heasman, 2006; Zuccotti et al., 2011). During oogenesis, massive transcription within the oocyte and from the supporting cells lead to accumulation and storage of messenger RNAs (mRNA) in the form of ribonucleoprotein (RNP) complexes. These mRNAs are essential for oocyte maturation and embryogenesis, during the period of transcription quiescence, before zygotic transcription begins.

1.1.1 Embryonic polarity, cell fate specification and axial patterning

Maternal factors play key roles in the establishment of polarity, cell fate specification and axial patterning by localizing to specific regions of the oocyte and the developing embryo before and after fertilization respectively (Dworkin and Dworkin-Rastl, 1990; Lasko, 1999; Lim et al., 2012; Lu et al., 2011; Martin and Ephrussi, 2009; White and Heasman, 2008). For example in sea urchins, maternal components required for posterior development are sequestered in the vegetal pole of developing oocytes. In sea urchin embryos, maternally provided β-catenin specifies the fate of vegetal micromeres and the levels of nuclear β-catenin accumulation in those cells determine mesodermal and endodermal cell fates (Davidson et al., 2002; Logan et al., 1999).

In *C. elegans*, maternally expressed *par* genes establish cellular and embryonic polarity. Upon fertilization PAR-2 and PAR-3 proteins localize to the posterior and anterior cortex respectively, specifying the anterior-posterior

polarity of the developing embryos (Goldstein and Macara, 2007; Noatynska and Gotta, 2012). PAR proteins later function in different contexts and also specify the apical basal polarity in epithelia (Nance, 2005). The transcripts of blastomere identity specifying genes like *mex-3, glp-1, skn-l* and *pie-1* are also maternally provided and they function downstream of *par* genes (Bowerman, 1995; Bowerman et al., 1997). MEX-3 and GLP-1 determine anterior cell fates, SKN-1 specifies intestine and muscle cell fates and PIE-1 is essential for totipotency of germ cells (Maduro et al., 2001; Mello et al., 1996).

In *Drosophila,* body axes are determined in the oocyte by regulated distribution of several maternal RNAs and proteins. The message for posterior determinant Gurken, is synthesized by nurse cells, transported to the oocyte, and then localized to the future posterior pole of developing oocytes (stage VII). Localized Gurken signaling renders posterior fate to a group of follicle cells. This leads to repolarization of the microtubule cytoskeleton in stage IX oocytes, following which gurken RNA and protein molecules move to the anterodorsal region with the oocyte nucleus. At this new location, a second wave of Gurken signaling instructs the adjacent follicle cells to acquire dorsal fates. The polarized microtubule cytoskeleton with more plus ends directed towards the posterior also leads to the localization of oskar and nanos RNA to the posterior by kinesin motors and bicoid RNA to the anterior by dynein motors (reviewed in (Cooperstock and Lipshitz, 2001; Kugler and Lasko, 2009; Lasko, 1999)). Maternal RNAs for anterior factor Hunchback and posterior factor Caudal are uniformly distributed along the anterior/posterior (A/P) axis. Localized Bicoid represses translation of caudal RNA in anterior and Caudal forms a gradient from posterior to anterior (Niessing et al., 2002). Similarly, localized Nanos represses translation of hunchback RNA in posterior and Hunchback forms a gradient from anterior to posterior (Sonoda and Wharton, 1999; Wreden et al., 1997). These morphogen gradients specify the cell fate in the *Drosophila* embryo along the A/P axis (Figure 1.1A).

Axis and tissue specification in *Xenopus* is also primarily driven by the asymmetric positioning of maternally deposited RNAs like *VegT*, *Wnt11* and

Ectodermin. *VegT* RNA is localized to the vegetal hemisphere of oocytes and encodes a T-box transcription factor. VegT induces endodermal transcription factors Xsox17 and GATA factors, and mesodermal transcription factor Mixer in vegetal and equatorial cells but not in animal pole cells. Hence, in the absence of VegT, ectodermal cell fates are expanded. Furthermore, ectoderm is specified by maternal RING-like ubiquitinase, Ectodermin which regulates both BMP and Activin-type signaling. Translocation of vegetally localized maternal *Wnt11* mRNA and Dishevelled protein to one side of the embryo during cortical rotation (Figure 1.1B), specifies the dorsal-ventral (D/V) axis of *Xenopus* embryos by activating canonical Wnt signaling pathway (reviewed in (Heasman, 2006; White and Heasman, 2008)).

Similar to *Xenopus*, the first asymmetry in zebrafish occurs before fertilization by the establishment of animal-vegetal (A/V) polarity. In zebrafish, A/V axis is specified in the developing oocytes when Balbiani body, an organelle composed of mitochondria, endoplasmic reticulum, germinal vesicles and several germplasm RNAs, is formed in the presumptive vegetal side. Maternal protein Bucky ball (Buc) is essential for formation of Balbiani body as well as vegetal localization of several RNAs (Abrams and Mullins, 2009; Bontems et al., 2009; Marlow and Mullins, 2008). Dorsal determinants are also first sequestered in the vegetal pole and upon egg activation translocate to the future dorsal via asymmetric parallel microtubule arrays (Jesuthasan and Stahle, 1997; Mizuno et al., 1999; Tran et al., 2012). Maternally deposited transcripts encoding Wnt8a are asymmetrically localized in the yolk of 4-8 cell stage zebrafish embryos and activate Wnt/β-catenin signaling pathway required for dorsal specification (Lu et al., 2011). Maternal transcripts encoding the Nodal-related factor Squint are asymmetrically localized in the blastoderm at the 4-cell stage and specify dorsal by a non-coding function that is dependent on the Wnt/β-catenin signaling pathway (Gore et al., 2005; Lim et al., 2012).

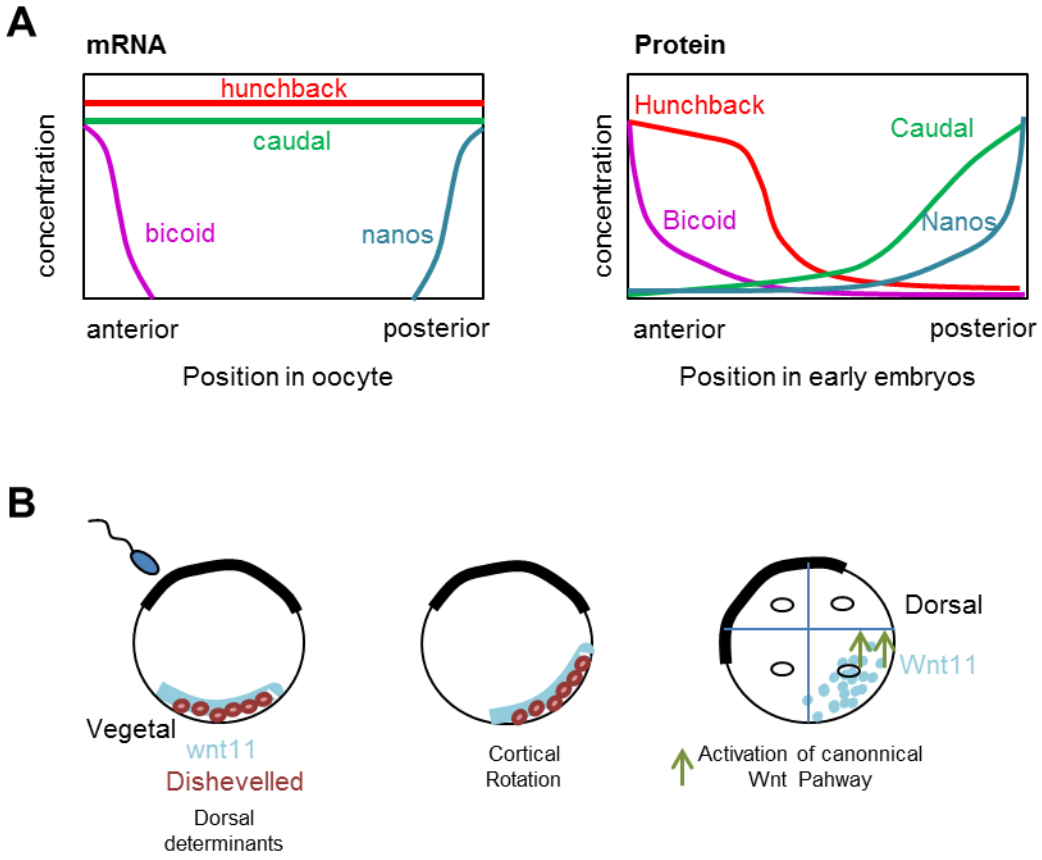


Figure 1.1- *Establishment of embryonic axis*

(A) Schematic depicting establishment and patterning of anterior-posterior (A/P) axis in *Drosophila.* In oocytes, bicoid and nanos RNA are localized to anterior and posterior poles respectively. RNAs encoding patterning proteins Hunchback and Caudal are distributed uniformly. Localized RNAs result in a gradient of Bicoid and Nanos proteins in the early embryos. Bicoid represses translation of caudal RNA so Caudal forms a gradient from posterior to anterior. Nanos represses translation of hunchback RNA so Hunchback forms a gradient from anterior to posterior. Gradients of these transcription factors pattern the A/P axis of *Drosophila* embryos. (B) Schematic depicting establishment of dorsal-ventral (D/V) axis in *Xenopus* embryos. Maternal dorsal determinants like Dishevelled protein and wnt11 RNA are deposited at the vegetal pole of oocytes. Upon sperm entry, the embryo undergoes cortical rotation leading to translocation of these determinants to the region opposite to sperm entry point. Wnt11 activates canonical Wnt signaling pathway in the cells receiving these determinants and leads to dorsal specification. Adapted from (Tao et al., 2005)

Mammalian embryos were previously thought to undergo regulative development as cell-fates are not determined during early cleavage stage (Ciemerych et al., 2000; Johnson and McConnell, 2004; Yamanaka et al., 2006) but some recent reports suggest that maternal factors can control cell lineage specification. Increased levels of maternally deposited *Cdx2* mRNA in vegetal blastomeres of mouse embryos, may contribute to trophectodermal cell fate (Bischoff et al., 2008; Jedrusik et al., 2008). Sub-cortical maternal complex (SCMC) proteins like MATER, FLOPED, PADI6, TLE6 and FILIA also segregate to outer cells of morula and these cells preferentially form trophectoderm rather than inner cell mass of the blastocyst (Johnson and McConnell, 2004).

1.1.2 Germline Specification

Across metazoans, germline cell fate is specified by two mechanisms – epigenesis (inductive signals) and preformation (maternally inherited determinants) (Figure 1.2) Mammalian embryos exhibit regulative formation of germ cells. In mouse embryos, a small number of pluripotent cells in the epiblast express germline competence genes and differentiate into primordial germ cells (PGCs), following inductive signals from the neighboring cells (Extavour and Akam, 2003). Hence, this mechanism of epigenesis does not require any known maternal contribution. However, in many other animal species, germline development takes place by the mechanism of preformation and localized maternal mRNAs play important roles in germplasm specification (Amikura et al., 2005; Raz, 2003; Schisa et al., 2001). Germplasm is a specialized cytoplasm, containing electron dense granules, many mitochondria and specific RNAs. Germplasm is segregated during early oocyte/embryonic development and the cells that acquire the germplasm become the PGCs. Maternally regulated localization of conserved RNAs, such as vasa, nanos and dazl, is crucial for germ cell formation in many organisms including *Drosophila*, *Xenopus* and zebrafish (Ewen-Campen et al., 2010; Saffman and Lasko, 1999).

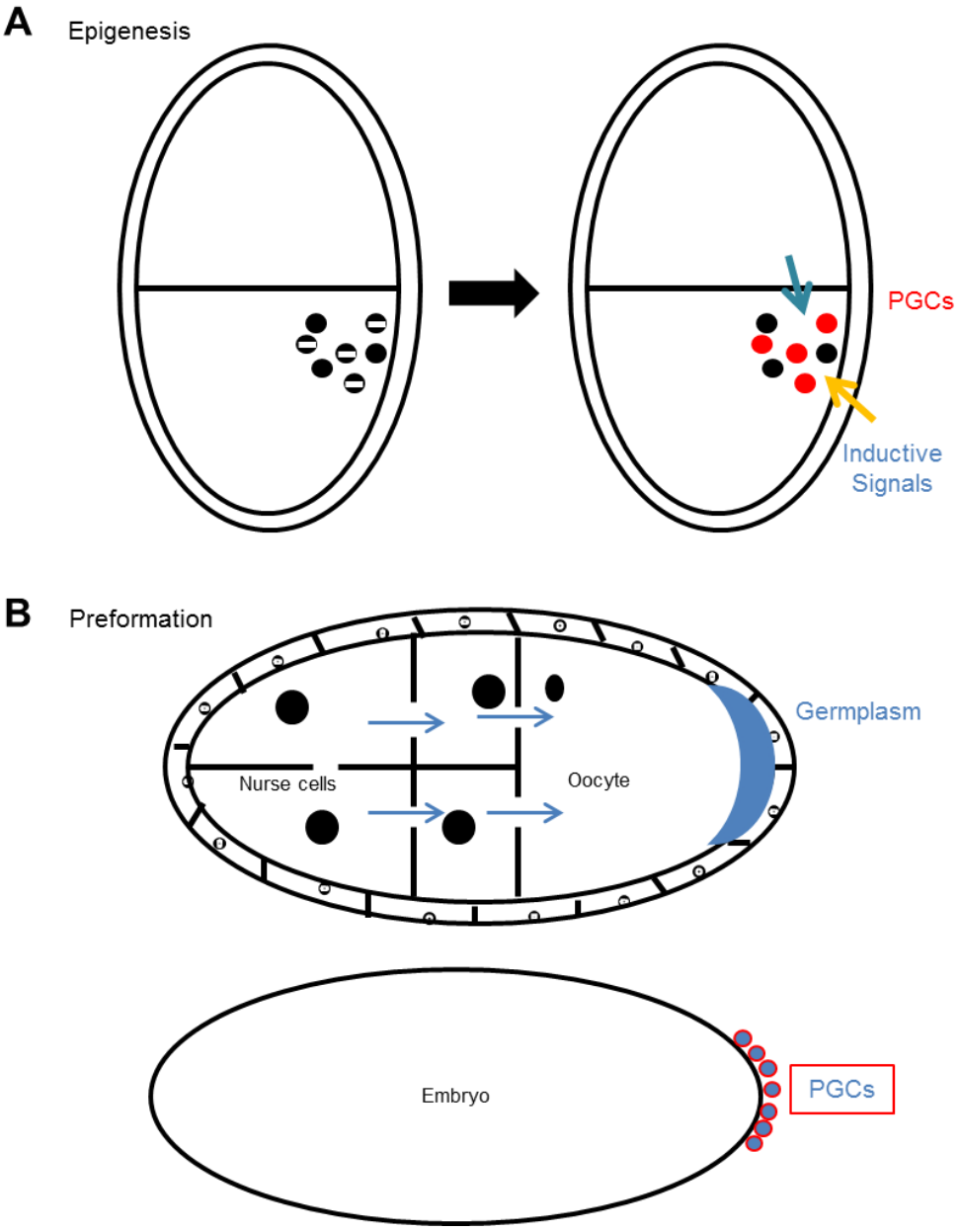


Figure 1.2 – *Models of germ cell specification*

(A) Epigenesis - In mammalian embryos, germplasm is not maternally provided. In mouse embryos, a group of pluripotent epiblast cells express germline competence gens (striped cells). These cells receive inductive signals (blue and yellow arrows) from neighboring tissue and become PGCs (red). (B) Preformation – During *Drosophila* oocyte maturation, germline determinants are produced by nurse cells and actively transported to the oocyte. These determinants localize to the posterior pole of oocytes and form poleplasm or germplasm (blue). The cells that acquire germplasm during cell divisions become the primordial germ cells (PGCs) as shown in the lower panel. Adapted from (Extavour and Akam, 2003)

Posterior localization of maternal oskar, vasa and nanos RNAs, in *Drosophila oocytes,* is essential for pole plasm (germplasm) assembly prior to the formation of pole cells during embryogenesis. Pole cells are the precursors of PGCs (Mahowald, 2001). During *Xenopus* oogenesis, electron dense cytoplasm (germplasm) containing germline determinants assemble at the vegetal cortex of the oocyte. Upon fertilization the germplasm gets segregated into 4 vegetal cells and thereafter is distributed unequally between daughter cells till MBT. The cells that acquire germplasm differentiate into PGCs (King et al., 2005). In zebrafish oocytes, RNAs and proteins required for germline development are segregated first into the vegetal Balbiani body and then localized by different pathways during oogenesis. Upon fertilization, maternally inherited germplasm components, including vasa, dazl and nanos, accumulate at the cleavage furrows during the first few cleavage cycles. During late blastula stages, four clusters of cells acquire the germplasm and become PGCs and migrate to the developing gonad during gastrulation (Kosaka et al., 2007; Raz, 2003).

1.1.3 Genomic imprinting and chromatin remodeling

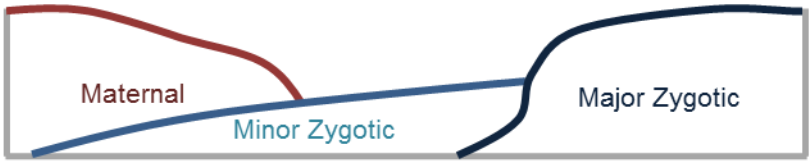
Maternally deposited proteins are also essential for various regulatory functions such as maintenance of genomic imprinting, reprogramming of maternal and paternal genomes (Chung et al., 2003; Howell et al., 2001; Ratnam et al., 2002), chromatin remodeling (Bultman et al., 2006; Burns et al., 2003), genome activation and oocyte to embryo transition (Tong et al., 2000; Wu et al., 2003). Maternal genome methylation is maintained by multiple DNA methytransferase (Dmnt) proteins that are expressed in mouse ovary. Dmnt3a and Dmnt3b establish maternal methylation during oogenesis. Dnmt1o, an oocyte specific Dmnt, and Dnmt3s regulate maternal imprinting in embryos (Hirasawa et al., 2008; Howell et al., 2001). Dapp3 (PGC7), a DNA/RNA binding protein preserves methylation of imprinted maternal loci and maternal mutants arrest prior to 2-cell stage due to cleavage failure (Nakamura et al., 2007). Repression and activation of zygotic genome is regulated by chromatin remodeling. Maternally deposited histones mediate repression until zygotic genome activation (ZGA). This repression is later

relieved via histone modifications like acetylation (Aoki et al., 1997; Schubeler et al., 2004; Vastenhouw et al., 2010). Maternal Stem loop binding protein (SLBP) regulates the stability and translation of Histone encoding RNAs and maternal mutants for SLBP arrest due to chromosomal and spindle defects (Kodama et al., 2002; Lanzotti et al., 2002). Hence, maternal factors regulate various facets of reprogramming required for epigenetic regulation and zygotic genome activation.

1.1.4 Maternal to zygotic transition (MZT)

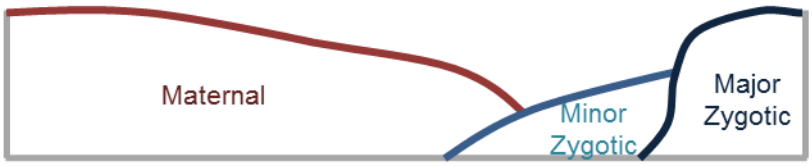
An embryo is largely under maternal control before zygotic transcription begins and gene products deposited by the mother execute early developmental events. The maternal program also initiates the degradation of maternal RNAs and proteins, an event that is critical for a successful transition from maternal to zygotic control. The developmental stage and timing of maternal to zygotic transition (MZT) vary in different species (Figure 1.3) and the proportion of maternal factors that degrade at MZT is also variable (Schier, 2007; Tadros and Lipshitz, 2009). Maternal and zygotic degradation activities were distinguished in *Drosophila* as egg activation and fertilization are two independent events. So, in activated eggs, only those RNAs and proteins which are regulated by maternal factors like Nanos, Hsp83 etc get degraded (Bashirullah et al., 1999; Walser and Lipshitz, 2011). In addition, a genome wide study using microarray also showed that a conserved multi-functional post-transcriptional regulator, Smaug (maternal) is a major regulator of maternal transcript destabilization (Tadros et al., 2007). Micro RNA (miRNA) mediated regulation of maternal RNAs is dependent on maternally provided Dicer (Murchison et al., 2007). Maternal proteins are also degraded at MZT by the ubiquitin-proteasome pathway, components of which are maternally expressed (Evsikov et al., 2006).

Sea urchin (*S. purpuratus*)



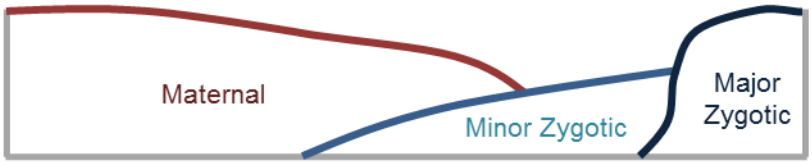
Time (hours)	0	2	15	30
Cleavage cycle	0	2	~8	~10

Nematode (*C. elegans*)



Time (hours)	0	0.7	1.25	3-3.5
Cleavage cycle	0	1	2	6-7

Fruit fly (*D. melanogaster*)

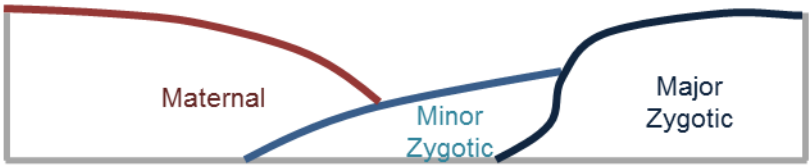


Time (hours)	0	1	1.5	2.5
Cleavage cycle	0	8	10	14

Zebrafish (*D. rerio*)

Time (hours)	0	2	2.75	5.25
Cleavage cycle	0	6	10	14

Mouse (*M. musculus*)



Time (hours)	0	10	22	37
Cleavage cycle	0	0	1	2

Figure 1.3 – *Maternal to Zygotic transition (MZT) in various model organisms*

Red curves represent degradation profile of maternal RNA whereas light and dark blue curves illustrate the minor and major waves of zygotic gene activation in each organism. Time in hours post fertilization and cleavage cycle is demarcated in the bottom. Adapted from (Tadros and Lipshitz, 2009)

Hence, during maternal to zygotic transition, maternally deposited RNAs and proteins that are no longer required or may be required only in a subset of cells are destroyed. In conjunction, there is reprogramming of gene expression for generation of transcripts that are not expressed in oocytes for further developmental processes. After ZGA, some maternal gene products, may still persist and cooperate with zygotic gene products for normal development.

1.2 Regulation of maternal RNAs

A large number of transcripts are expressed in oocytes. Some of them are required for oocyte maturation while others are stored in the form of mRNP and are translated and/or degraded in an orchestrated manner during the early phases of embryonic development. The duration of oogenesis in animals can vary from a few days, as in *Drosophila*, to several years, as in humans. Maternally deposited RNAs are under tight post transcriptional control during oocyte maturation and early embryogenesis (Bashirullah et al., 2001; Bettegowda and Smith, 2007; Johnstone and Lasko, 2001; Meric et al., 1996; Tadros and Lipshitz, 2005) (Figure 1.4). Drosophila oogenesis and embryogenesis have proved to be excellent systems to study regulation of maternal RNAs as spatiotemporal organization of maternally deposited RNAs in the egg is critical for correct patterning of the embryo. Nearly 70% of RNAs are localized in several different patterns during Drosophila oogenesis and early development (Lecuyer et al., 2007). Although mechanism by which all these RNAs are localized has not been characterized, the regulation of bicoid, gurken and oskar mRNAs in the oocytes has been studied extensively (Bashirullah et al., 1998; Lasko, 1999).

1.2.1 RNA localization

In recent years, RNA localization has emerged as an important process in cell and developmental biology. RNA localization is a very common as well as efficient mechanism for gene product distribution to specific locations in cells and embryos (Medioni et al., 2012). As we can imagine, a single RNA molecule can

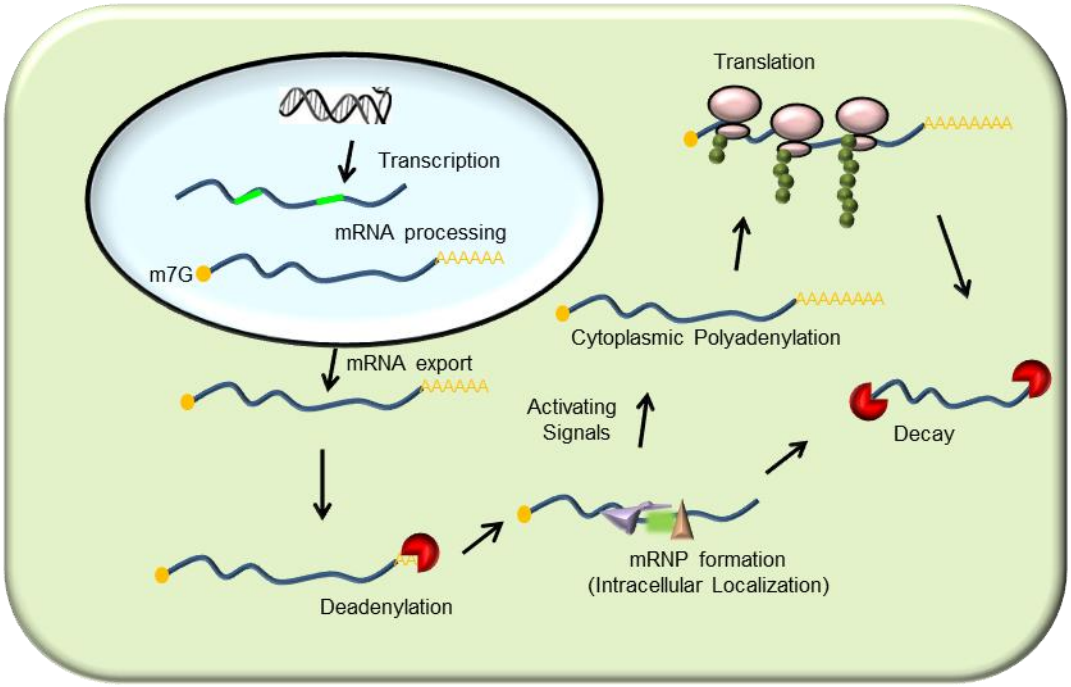


Figure 1.4 – *Regulation of maternal RNAs*

Maternally deposited mRNAs require additional steps of processing for stabilization and storage before localization and/or translation. After transcription, mRNAs undergo processing within the nucleus and 5' 7methyl guanosine (m7G) cap and 3' polyA tail are added. Introns are removed by splicing. Processed mRNAs are exported to cytoplasm where they undergo regulated deadenylation. Several RNA binding proteins and associated proteins recognize the mRNAs and form mRNP complexes. Such mRNP complexes can be localized to specific compartments or stored in the cytoplasm. Developmental cues can either signal for decay of these mRNAs or activate translation by cytoplasmic polyadenylation.

be translated into many protein molecules. Therefore, RNA localization may be preferred over protein localization as it is more energy efficient. As discussed earlier, cell fate specification and patterning during embryonic development requires generation of polarity which is achieved by action of signaling proteins in specific compartments. Spatial mis-expression of such proteins can lead to catastrophic effects. For instance, ectopic expression of Nanos or Oskar in the anterior region of *Drosophila* embryos disrupts the A/P axis and leads to the formation of two posterior structures which are mirror images of each other (Gavis and Lehmann, 1992; Yoshida et al., 2004). RNA localization also provides temporal control and fine tuning of gene expression. In response to developmental cues, localized RNAs are better poised for rapid translation as compared to activation of *de novo* gene expression, protein synthesis and localization of protein molecules (Besse and Ephrussi, 2008; Martin and Ephrussi, 2009). Also, different isoforms of RNA can be localized to distinct cellular compartments and lead to different downstream events (Baj et al., 2011).

Apart from the establishment of embryonic polarity and patterning, RNA localization facilitates many other cellular processes. RNA localization is crucial for co-translational assembly of macromolecules at the right place. Many cytoskeletal proteins assemble during the translation of nascent peptides (L'Ecuyer et al., 1998; Singer, 1992). An elevated level of β Actin, required in the lamellipodia of migrating fibroblasts, is achieved by the localization of β actin mRNA to the leading edges of fibroblast (Condeelis and Singer, 2005; Lawrence and Singer, 1986). Messenger RNA localization is also very important in neuronal cells for the expression of synaptic proteins, in response to stimuli received at the tip of the axons and dendrites (Mikl et al., 2010; Roegiers, 2003). In yeast the localization of *ASH1* to the daughter cell is required for mating-type switching during budding (Bobola et al., 1996). In *Drosophila* localization of RNAs like hairy, wingless, unpaired etc is required for apical-basal polarity of epithelial cells (Bullock et al., 2004; Simmonds et al., 2001).

1.2.2 Cellular machinery for RNA localization

Some of the mechanisms implicated in RNA localization include localized protection from degradation, diffusion coupled with localized entrapment and active transport along a polarized cytoskeleton (Figure 1.5) (Bashirullah et al., 1998; Jansen, 2001; Medioni et al., 2012). For example, asymmetric accumulation of hsp83 RNA at the posterior pole of *Drosophila* embryos is achieved by localized protection against a widespread degradation (Semotok et al., 2005). Also, posterior localization of nanos in *Drosophila* embryos requires RNA decay in the rest of the cytoplasm (Bergsten and Gavis, 1999). In *Xenopus* oocytes, germplasm RNAs, *Xcat2* and *Dazl1* are reported to localize by diffusion and entrapment by dense endoplasmic reticulum network in the vegetally localized mitochondrial cloud (Chang et al., 2004). From a vast body of research in the RNA localization field, active directed transport seems to be the predominant mechanism and is observed in many cell types including oocytes/embryos, neurons, migratory cells and epithelial cells. This is a multi-step process which first requires the assembly of a localizing granule by binding of RNA to *trans* factors (RNA binding proteins) that recognize specific *cis* elements in the RNA. Such mRNP complexes can then recruit the motor proteins that move along the cytoskeleton. After reaching the destination, mRNA is anchored to prevent diffusion (Wilhelm and Vale, 1993).

ZIPCODES – This term was coined by Robert H. Singer to describe the *cis*-elements in the transcripts that are required for localization (Singer, 1993). Mutations in *cis* elements severely affect the localization process and *cis* elements can confer localization when fused to any heterologous sequences. The *cis* elements are utilized by different mechanisms of RNA localization. These elements are most often (but not exclusively) found in the 3'UTR of transcripts and can contain sequence and/or structure information. The length of such elements can vary from a few nucleotides to several hundred bases and can be either discrete or redundant. For instance, a 44 nucleotides signal in the 3'UTR of *K10* is necessary and sufficient for transportation from nurse cells to oocyte as well as its anterior localization in *Drosophila* oocytes (Serano and Cohen, 1995).

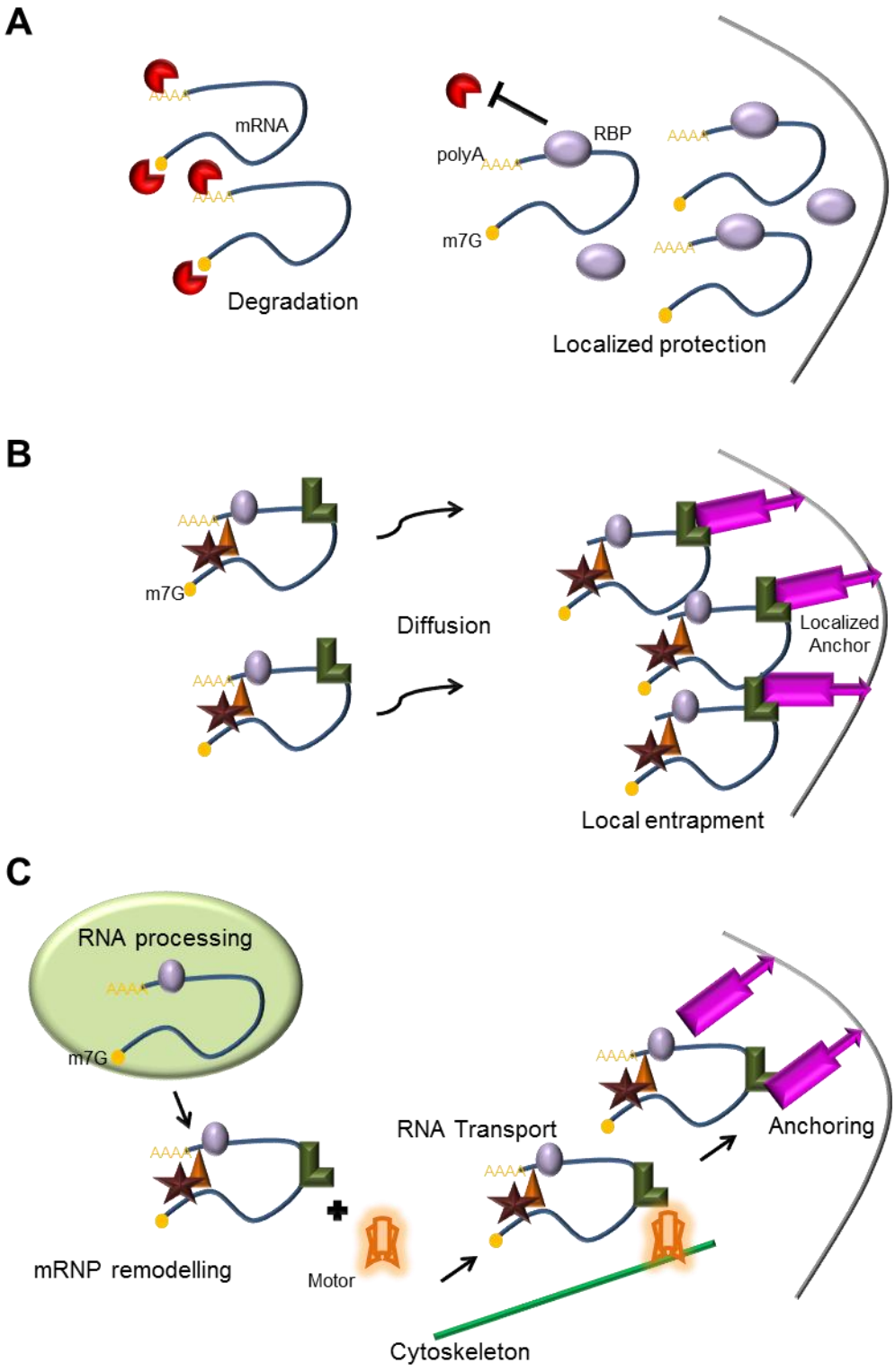

A
mRNA
AAAA
Degradation
RBP
polyA
m7G
Localized protection
B
m7G
Diffusion
Localized
Anchor
Local entrapment
C
RNA processing
m7G
mRNP remodelling
Motor
RNA Transport
Anchoring
Cytoskeleton

Figure 1.5 – *Mechanisms underlying mRNA localization*

(A) Localized protection from degradation – mRNA molecules that do not sequester to a specific subcellular compartment are degraded. (B) Diffusion coupled with localized entrapment – mRNAs diffuse freely in the cytoplasm and are entrapped by a localized anchor. (C) Active localization – mRNAs that are actively transported are recognized by specific *trans*-acting factors in the nucleus and after export to the cytoplasm the mRNP complex is remodeled and certain factors load the localizing granule on molecular motors that move along polarized cytoskeleton. At the destination, mRNAs are anchored to prevent diffusion. Adapted from (Medioni et al., 2012)

In contrast, multiple elements in bicoid 3'UTR are required for different steps of its localization in *Drosophila* oocytes. A 50 nucleotide stem loop, BLE1 is necessary and sufficient for transport from nurse cells to oocyte while stems IV and V function in anterior localization of bicoid within the oocyte cytoplasm and stem III is required for anchoring (Ferrandon et al., 1994; Macdonald and Kerr, 1997; Macdonald et al., 1993). Similarly, distinct elements are responsible for different stages of localization of oskar RNA. The element required for accumulation of oskar in the oocytes lie between nucleotides 532-791 of the 3'UTR. Other *cis*-regulatory elements required for release of oskar RNA from the anterior pole reside in two smaller regions, nucleotides 242-363 and nucleotides 791-846, in the 3'UTR. Posterior localization is mediated by *cis*-elements present in nucleotides 1-242 of the 3' UTR (Kim-Ha et al., 1993). In addition, splicing dependent localization has been observed in the case of oskar RNA, where assembly of the exon-junction complex (EJC) is required for localization (Ghosh et al., 2012; Hachet and Ephrussi, 2004). Elements for gurken localization are present in the 5' and 3' UTRs, as well as the coding region. Nucleotides 1-35 of the gurken 5'UTR, named as gurken localization element 1(GLE1), is important for stable localization during early and mid-oogenesis. During this period gurken RNA accumulates within the oocytes. The final stage of gurken localization to the anterodorsal corner of oocytes is mediated by the elements in the 3'UTR (Saunders and Cohen, 1999; Thio et al., 2000). *Vg1,* a vegetally localized RNA in *Xenopus* oocytes has multiple but redundant localization elements (VM and E2 motifs) in the 3' UTR (Lewis et al., 2004). Although many localized RNAs are known, the lack of well characterized RNA recognition motifs limits the in-depth understanding of *cis* elements. One of the reasons for this is redundancy in localization elements, as well as, the use of multiple elements by the same RNA. In addition, prediction of secondary and tertiary structure of localization motifs is difficult.

RBPs, Adaptors and Motors – RNA binding proteins are the *trans* factors that recognize localization signals in RNAs and by binding to them, initiate the assembly of the localizing granule. Such mRNPs may contain a large

number of associated proteins which may have multiple functions like RNA localization, stabilization and translational repression (Bashirullah et al., 1998; Martin and Ephrussi, 2009). In some cases, nuclear events are also important for cytoplasmic localization. RBPs can bind the target RNA during transcription or splicing and direct it to the cytoplasmic localization machinery (Marchand et al., 2012). Following RNA export, mRNPs undergo remodeling and bind to motor proteins which move along the cytoskeleton network. Several RNA binding proteins with roles in RNA localization have been identified. Purification of large localizing granules showed presence of several adaptor proteins in the complex that link the direct RNA binders to motor proteins (Elvira et al., 2006; Kanai et al., 2004). Specific localization of mRNP complexes is dependent on other proteins in the ternary complex. Molecular motors which move directionally along the cytoskeleton tracks are divided in three major classes – Kinesins (plus end directed) and Dyneins (minus end directed) move on microtubule tracks while Myosins travel on actin tracks (Vale and Milligan, 2000).

Localization of bicoid RNA to the anterior of *Drosophila* oocytes requires several maternal *trans*-acting factors such as Exuperentia, Swallow and Staufen. Staufen can bind to stem loop III, IV and V of bicoid 3'UTR and is required for the final stages of localization of bicoid RNA to the anterior of oocytes (Berleth et al., 1988; St Johnston et al., 1989; Stephenson et al., 1988). Stem loop IV and V in bicoid 3' UTR are also recognized by a protein complex containing the RNA binding proteins, Smooth, Modulo and PABP and, a Kinesin family motor protein, Nod (Arn et al., 2003). It has been suggested that Swallow is an adaptor protein that connects bicoid RNA to dynein motor (Schnorrer et al., 2000). Posterior localization of oskar RNA in *Drosophila* oocytes is dependent on several *trans* factors. Some genes which have been implicated in oskar localization are *cappuccino, spire, staufen, orb, mago nashi, notch,* delta and the maternal form of protein kinase A (PKA) (Gonsalvez and Long, 2012; Lasko, 1999). Orb directly binds to oskar RNA and plays important roles in its localization, anchoring, and translational activation (Chang et al., 1999; Christerson and McKearin, 1994). Staufen protein colocalizes with oskar RNA

throughout oogenesis and in *staufen* mutants oskar RNA transiently remains in the anterior (Micklem et al., 2000; St Johnston et al., 1991). Staufen also function in RNA localization in mammalian neurons (Tang et al., 2001). Vg1RBP binds to the 3'UTR of vg1 RNA and localizes the RNA to the vegetal pole of *Xenopus* oocytes (Zhang et al., 1999). Studies in chick embryo fibroblasts led to the identification of Zipcode binding protein1 (ZBP1) that binds and localizes β actin RNA (Farina et al., 2003).

In recent years, extensive progress has been made in the field of RNA localization. For better understanding of the diverse RNA sorting mechanisms, it is important to determine the precise molecular composition of mRNP complexes and uncover the mechanisms that regulate their metabolism. The complex composition of mRNP granules suggest that there are regulatory mechanisms involved in their assembly, which remain to be elucidated (Xing and Bassell, 2013).

1.2.3 Translational repression and RNA storage

Generally, mRNAs are polyadenylated in the nucleus and get translated in the cytoplasm after export from the nucleus. However, regulation of maternal RNAs requires additional steps of transcript stabilization, so that they can be translated at specific time points. Immediate translation is prevented by deadenylation of these RNAs in the cytoplasm. RNA binding proteins can stabilize such deadenylated RNAs by forming mRNPs. Exo-nucleolytic degradation of polyA tail can lead to either mRNA decay or silencing. The length of polyA tail at the 3' end of mRNA decides the translational potential of an mRNA and shortening of polyA tail correlates with translational repression (de Moor and Richter, 1999; Richter, 1999). Deadenylation as well as cytoplasmic polyadenylation prior to translation are mostly dependent on *cis*-regulatory elements in the 3'UTR of RNAs. The deadenylation signals include alternative polyadenylation signal (APA), AU-rich elements (ARE) and miRNA target sites (Zhang et al., 2010). A prominent example of a *trans* factor involved in deadenylation is Poly(A)-specific ribonuclease (PARN). PARN, a conserved

deadenylase is important for oocyte maturation and early development in *Xenopus* (Balatsos et al., 2012; Korner et al., 1998). The *cis* element EDEN and the binding protein, EDEN-BP also function in sequence specific deadenylation of eg5 and c-mos maternal RNAs in *Xenopus* embryos (Paillard et al., 1998). A short polyA tail significantly interferes with translation but may not be sufficient to completely block translation (Smith et al., 1988).

Maternal RNAs are stored in mRNP granules until they are translated. The binding of RNA binding proteins masks the mRNAs and protect them against degradation as well as blocks translation. RNA associated proteins such as Rap55, YBX2 (MSY2, FRGY2), Xp54 and PRMT1 localize to mRNP foci in the cytoplasm and package mRNAs (Murray et al., 1992; Yang et al., 2006). Storage of mRNAs in cytoplasmic granules is reversible and mRNA can exit from its repressed state and enter the state of active translation (Figure 1.6A) (Bhattacharyya et al., 2006; Brengues et al., 2005). In such mRNP granules, which are also known as P bodies or sponge bodies, the fate of an RNA is decided by specific regulatory proteins – either translation, silencing or decay (Eulalio et al., 2007). So, in addition to non-specific binders, sequence-specific proteins interacting with 3' and 5' untranslated region (UTR) are also important for mRNA masking (Spirin, 1994).

Translation is generally repressed at the initiation step. Translational initiation takes place by the assembly of the eIF4F complex at the 5' 7-methylguanosine cap and recruitment of 40s pre-initiation complex. Components of the eIF4F complex include eIF4E that recognizes the 5' cap structure, eIF4G that circularizes the RNA by interacting with polyA binding protein and recruits the ribosomal complex and eIF4A that functions as an RNA helicase (Gingras et al., 1999). Generally, translation initiation can be blocked by two kinds of proteins that interfere with eIF4F complex formation – eIF4E binding protein (4EBP) and eIF4E homology proteins (4EHP) (Figure 1.6B, C). In *Drosophila* oocytes, posteriorly localized oskar RNA is translationally repressed by Cup (a 4EBP) prior to localization. Cup regulates osk RNA by interacting with a RNA binding protein, Bruno which recognizes specific sequence motifs in osk RNA.

Cup competes with eIF4G for eIF4E binding and hence blocks translation (Nakamura et al., 2004a). Other proteins in osk mRNP that function in translational repression are Maternal expression at 31 B (Me31B) and Polypyrimidine tract binding protein (PTB) (Besse et al., 2009; Nakamura et al., 2001). Cup also regulates translation of nanos by interacting with a nanos binding protein, Smaug (Nelson et al., 2004). Smaug can also function by recruiting CCR4 deadenylase complex (Semotok et al., 2005). Other examples of 4EBPs that block translation initiation are Maskin and Pumilio. Maskin binds to Cytoplasmic polyadenylation element binding protein (CPEB) which recognizes *cis* elements in 3'UTRs known as CPE (Cao et al., 2010; Stebbins-Boaz et al., 1999). 4EHP is eIF4E related cap binding proteins that cannot bind to eIF4G and hence interferes with translation initiation. In *Drosophila* embryos, translation of Caudal is regulated by an RNA binding protein, Bicoid which interacts with 4EHP (Cho et al., 2005). Mammalian homolog of Bicoid, Prep1 also regulates translation of hox4B RNA by binding to 4EHP (Villaescusa et al., 2009).

1.2.4 Transport and translation are linked

Localization of mRNAs is often coupled with their translational regulation and it is important that the mRNA is not translated until the message is delivered to the correct site within the cell/tissue. The requirement of correct mRNA localization for translational activation is a widely used theme during embryonic development. This idea that the mechanisms of localization and translational regulation are not mutually exclusive emerged majorly from work in *Drosophila.* Many *Drosophila* mRNAs are specifically localized with the goal of producing a localized protein. During embryogenesis, disruption of localization of oskar and nanos RNAs blocks their translation (Gavis and Lehmann, 1994; Kim-Ha et al., 1995). Similarly, vg1 RNA in *Xenopus* oocytes is also translationally repressed prior to it vegetal localization (Otero et al., 2001). Hence, the localizing granules contain either silenced mRNAs and/or translational repressors, which are inactivated upon correct localization (Besse and Ephrussi, 2008).

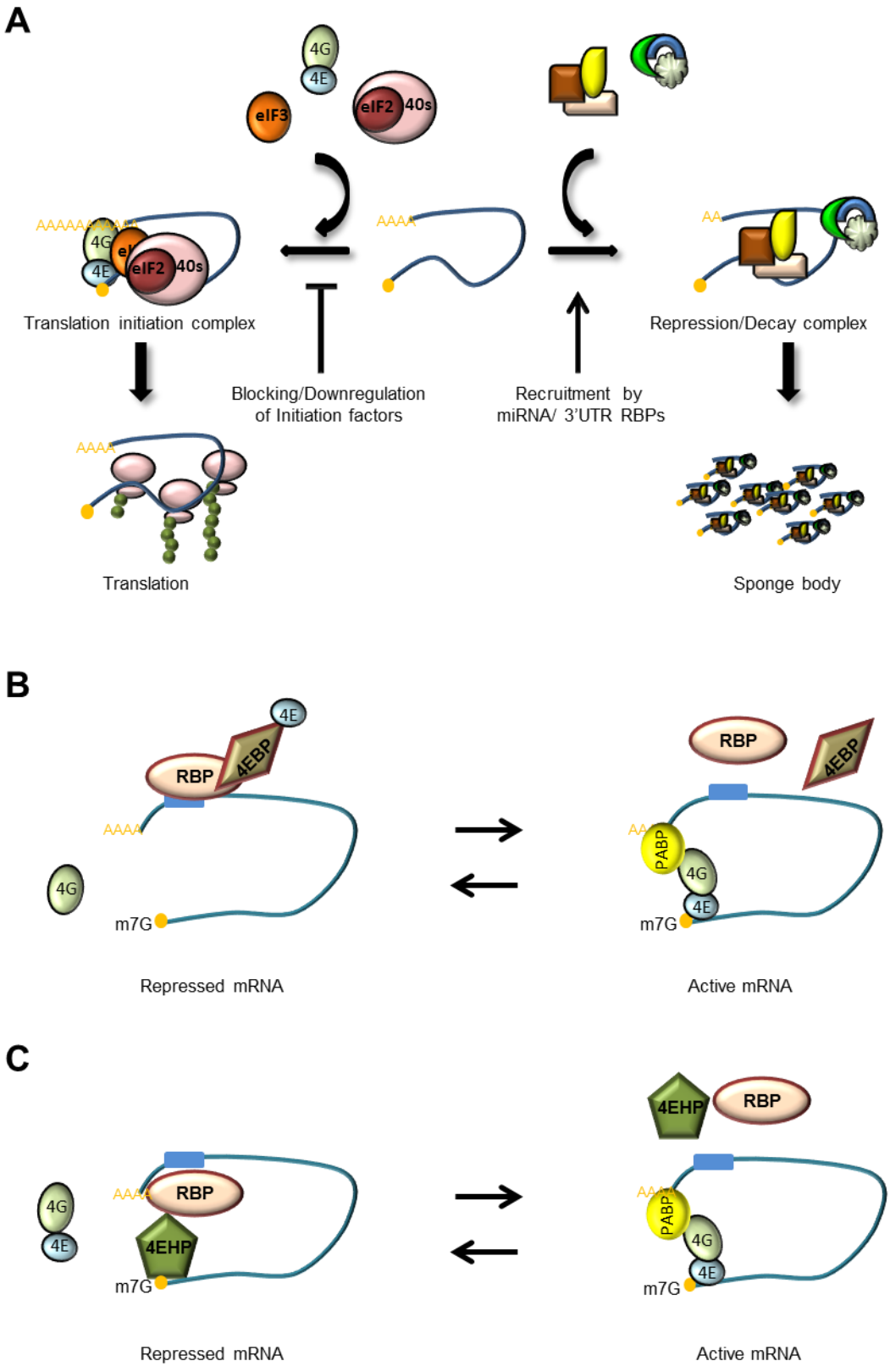

A
4G
4E
eIF3
eIF2
40s
AAAAAAAAAA
4G
4E
eIF2
40s
Translation initiation complex
AAAA
AA
Repression/Decay complex
Blocking/Downregulation
of Initiation factors
Recruitment by
miRNA/ 3'UTR RBPs
AAAA
Translation
Sponge body
B
4E
RBP
4EBP
AAAA
4G
m7G
Repressed mRNA
RBP
4EBP
AA
PABP
4G
4E
m7G
Active mRNA
C
4G
4E
AAAA
RBP
4EHP
m7G
Repressed mRNA
4EHP
RBP
AAAA
PABP
4G
4E
m7G
Active mRNA

Figure 1.6 – *Translational repression*

(A) Reversible recruitment of translation initiation complex or RNA decay/repression complex. mRNA when bound by translation initiation complex is actively translated while recruitment of degradation factors will lead to RNA decay. mRNA can also be stabilized and stored in repressed form in sponge bodies by formation of mRNP complexes. Adapted from (Parker and Sheth, 2007). (B) Translational repression by eIF4E binding proteins (4EBPs). A 4EBP when recruited by an RNA binding protein (RBP) competes with eIF4G for eIF4E binding and hence blocks translation. (C) Translational repression by eIF4E homology protein (4EHP). 4EHP compete with eIF4E for 5' cap binding, thereby blocks translation. The equilibrium of competitive bindings shown in B and C are dependent on the RBP. Adapted from (Richter and Lasko, 2011).

1.2.5 Translational activation of Maternal RNAs

Translation is initiated by formation of a closed loop when polyA binding protein (PABP) binds to the 5' cap binding proteins to assemble the translation initiation complex – eIF4F. The interaction between 3' and 5' UTR is mediated by eIF4G that can bind to both PABP and eIF4E. eIF4G-eIF4E interaction is critical for translation initiation (Hernandez and Vazquez-Pianzola, 2005). Circularization of RNA also facilitates re-initiation of translation once translation is terminated at the stop codon and it also protects RNA from degradation (Gingras et al., 1999; Mazumder et al., 2001).

As discussed earlier, stored mRNAs have short polyA tails so the unmasking can be accomplished by elongation of polyA tail. PABP is recruited by elongated polyA tail. Two *cis* regulatory elements have been implicated in cytoplasmic polyadenylation – the U rich cytoplasmic polyadenylation element (CPE) and the consensus hexanucleotide AAUAAA (Oh et al., 2000; Proudfoot and Brownlee, 1976; Wickens and Stephenson, 1984). CPE is bound by a 62 kDa protein, CPEB during the dormant phase (Kim and Richter, 2006). In response to specific cell cycle dependent stimuli, MAP kinase phosphorylates CPEB. This phosphorylation event recruits cleavage-polyadenylation specificity factor complex (CPSF complex) and the associated polyA polymerase, resulting in polyadenylation and translation of maternal RNAs (Fox et al., 1992; Keady et al., 2007; Murthy and Manley, 1995).

Translation of localized RNAs is also actively repressed during transport and the repression is alleviated upon correct localization. This can be achieved by the binding of repressors to other partner proteins which are expressed at the destination. For example, translation of nanos is repressed by Smaug and interaction of Smaug with Oskar protein at the posterior pole leads to translation of nanos mRNA (Dahanukar et al., 1999; Zaessinger et al., 2006).

The above description of translation activation is incomplete as maternally deposited RNAs are recruited for translation in a stage specific manner after fertilization. This requires additional mechanisms to prevent translation of a sub-population of RNA required at later stages and their stage specific recruitment

(Potireddy et al., 2006). Hence, this suggests that additional novel *cis* and *trans* factors function in a combinatorial manner for stage specific translation regulation during embryonic development (Pique et al., 2008; Tremblay et al., 2005).

1.3 Zebrafish as a model organism

Zebrafish is a small fresh water fish belonging to the group of cyprinid teleost. Laboratory techniques for zebrafish husbandry are well established for both adults and juveniles (Westerfield, 2007). George Streisinger and colleagues first identified the advantages of zebrafish for laboratory research (Streisinger et al., 1981). Zebrafish exhibits high fecundity and external fertilization, so very early developmental stages are readily accessible for analysis. The early embryo is transparent which allows microscopic examination of cellular and developmental processes with great detail. In addition, zebrafish embryonic development is rapid and by 3 days post fertilization, the embryo develops into a freely swimming larva and several organ systems are functional (Stuckenholz et al., 2004). In 3-4 months zebrafish becomes sexually mature and can generate new offspring. Owing to its popularity as a model organism, many laboratories worldwide have studied zebrafish development in substantial depth and detailed literature is available. Most of the information is electronically catalogued in a searchable format (http://zfin.org) (Sprague et al., 2001).

Zebrafish emerged as a widely accepted genetic tool for studying vertebrate development and disease in the 1990s when large scale forward mutagenesis screen were conducted (Driever et al., 1996; Haffter et al., 1996). These screens identified a large number of genes having essential functions during embryonic development. Random mutagenesis approaches, such as ENU, gamma rays, retroviral and transposon insertions have been used for nearly two decades. However, in the last few years, targeted knock outs were made possible with the advancement in targeted nucleases technology. Zinc finger nucleases, TALENs (transcription activator-like effector nuclease)and CRISPR (Clustered, regularly interspaced, short palindromic repeat)/cas9 have been used successfully to create targeted lesions in the zebrafish genome (Doyon et al., 2008; Huang et

al., 2011; Hwang et al., 2013; Lim et al., 2013; Meng et al., 2008). More recently, even homologous recombination has been reported though the efficiency remains low (Zu et al., 2013).

1.3.1 Maternal effect studies

The earliest stages of embryonic development are regulated by maternally provided gene products. Therefore, the study of maternal effect is of utmost importance. Zerbafish is an excellent model for the study of maternal contribution to embryonic development as eggs are externally fertilized. To study the earliest events even oocytes can be manipulated, followed by *in vitro* fertilization (Gore et al., 2005; Nair et al., 2013). In addition, the technique of PGC transplant is well established in zebrafish (Ciruna et al., 2002). Therefore, the role of maternal effect genes with essential zygotic functions (embryonic lethal) can still be investigated. Lastly, in zebrafish, the major wave of zygotic transcription begins only after 3 hpf, unlike that in mouse embryos (Kane and Kimmel, 1993). Hence, there is ample time for the functional analysis of maternal factors, without interference from zygotic gene expression.

A number of maternal-effect mutations have been identified in specialized screens (Dosch et al., 2004; Pelegri et al., 2004; Pelegri and Mullins, 2004; Wagner et al., 2004). Some of these mutants are listed in Figure 1.7. These maternal mutants have proved to be very useful for the study of maternally controlled processes, many of which are still poorly understood (Abrams and Mullins, 2009). As discussed earlier, maternally provided transcripts are under tight post transcriptional regulation which includes mRNA localization, stability, processing and translational regulation. Zebrafish embryos prove to be an excellent system to study RNA metabolism. *In situ* RNA hybridization to study spatial distribution of RNAs is used widely in zebrafish (Howley and Ho, 2000; Kudoh et al., 2001). In addition, fluorescently labeled RNA can be injected and tracked by live imaging (Gore et al., 2005; Tran et al., 2012). Transgenic lines can be established to manipulate localization and translational control elements (Yasuda et al., 2010). High throughput RNA sequencing and ribosome profiling

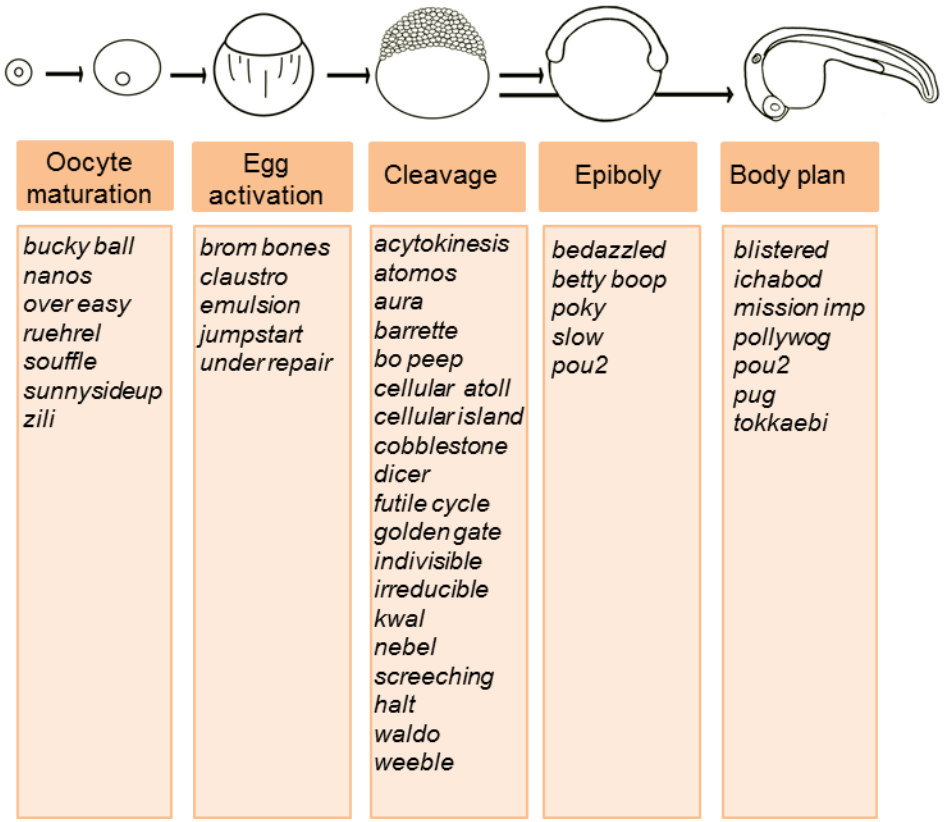


Figure 1.7 – *Maternal effect genes affecting zebrafish development at different stages.* Adapted from (Abrams and Mullins, 2009)

can be performed to study RNA stability, processing and translation (Aanes et al., 2011; Chew et al., 2013).

1.3.2 Zebrafish development and dorsal specification

In the Sampath laboratory, one of the major interests is to understand how maternally deposited factors establish polarity in oocytes/embryos and specify embryonic axes. We are studying these events by using zebrafish as a model system for reasons aforementioned. Zebrafish embryogenesis begins with sperm entry. When the oocyte is fertilized yolk to blastoderm cytoplasmic streaming begins and at the 1-cell stage a clear blastodisc is formed at the animal pole, on top of the yolk. The first cleavage occurs at 40 minutes post fertilization (mpf) and after this the embryo undergoes synchronous cell divisions during the next 3 hours. At 3 hours post fertilization (hpf), after the 10^{th} cell division cycle, the major wave of zygotic transcription is initiated. The zygotic genome is activated once continual cell divisions increase the DNA:cytoplasm ratio to a critical threshold (Kane and Kimmel, 1993). Gastrulation begins at 4hpf with a process known as epiboly, wherein the cells in blastoderm migrate towards the vegetal pole spreading over the whole yolk by 10hpf. During gastrulation, the cells at the margin between blastoderm and yolk internalize and convergent-extension movements also take place. This results in formation of the shield (organizer) which is observed as thickening of cells at the dorsal blastoderm margin (Solnica-Krezel, 2005). The shield defines dorsal and is the first morphological structure that breaks an otherwise radially symmetrical embryo. However, several embryological experiments showed that events required for dorsal specification take place before the first cell division and dorsal determinants are maternally deposited at the vegetal cortex (Mizuno et al., 1999; Ober and Schulte-Merker, 1999). These determinants are transported to future dorsal by microtubules in the first 30 mpf (Jesuthasan and Stahle, 1997; Tran et al., 2012).

Identification and analysis of maternal effect mutants like *ichabod, tokkaebi* and *hecate* showed that dorsal is specified by Wnt/β-catenin signaling (Kelly et al., 2000; Lyman Gingerich et al., 2005; Nojima et al., 2010). Maternal β

catenin is expressed throughout the blastoderm but localizes to the nucleus only in a few dorsal marginal cells, in response to Wnt signaling. The nuclear localization of β catenin initiates the dorsal program (Schneider et al., 1996). Recent studies showed that maternal wnt8a mRNA is asymmetrically localized at the vegetal cortex in 2-8 cell stage embryos, and likely activates Wnt signaling (Lu et al., 2011). However, the expression domain of wnt8a RNA in vegetal yolk is very wide. Therefore, it is unclear as to how Wnt8a activity is restricted to only 3-4 cells at the 128-cell stage. Another important factor required for nuclear localization of β catenin and specification of dorsal is maternal squint (sqt) RNA. Maternal sqt specifies dorsal by a non-coding activity of the RNA. It is speculated that sqt transcripts act as a scaffold to deliver dorsal determinants to the right location (Lim et al., 2012). Taken together, some of the key molecules involved in dorsal initiation have been defined (Langdon and Mullins, 2011; Lim et al., 2012; Lu et al., 2011). However, a major question that remains unanswered is how Wnt and Nodal/Squint pathway components cooperate to limit Wnt signaling to a small cluster of cells in the early blastula.

1.3.3 Role of maternal squint in dorsal specification

Squint is a Nodal-related signaling molecule belonging to the transforming growth factor beta (TGFβ) superfamily. Nodal signaling plays important roles during embryonic development with essential functions in axis specification and germ layer patterning in sea urchins, snails, ascidians, frogs, fish, and mammals (Collignon et al., 1996; Constam, 2009; Duboc et al., 2010; Erter et al., 1998; Feldman et al., 1998; Grande and Patel, 2009; Hudson and Yasuo, 2005; Jones et al., 1995; Rebagliati et al., 1998; Sampath et al., 1998; Shen, 2007). In addition, Nodal signaling has also been shown to be required for the maintenance of undifferentiated human and mouse ES cells (James et al., 2005; Vallier et al., 2005). Mis-regulated Nodal signaling has been found associated with tumor metastases (Topczewska et al., 2006).

Zebrafish has three Nodal-related ligands: Squint (Ndr1), Cyclops (Ndr2) and Southpaw. Nodal ligands signal by binding to the type I (ActRIB/Acvr1b)

and type II serine-threonine kinase receptors (ActRIIA/Acvr2a or ActRIIB/Acvr2b) (Figure 1.8) (reviewed in (Schier, 2003; Schier, 2009b; Shen, 2007). Unlike other TGFβ ligands like Activin, Nodal ligands cannot signal in absence of EGF-CFC co-receptors (One-eyed pinhead (Oep)). In the extracellular space, several inhibitors can regulate Nodal signaling. Lefty proteins can antagonize Nodal signaling by binding to the ligands or the co-receptor (Oep). Lefty generally functions downstream of Nodal signaling hence, forming a feedback regulation mechanism. Members of the Cerebrus family can also block signaling by interacting directly with the ligands. Downstream of type I and type II receptors, Nodal signaling is transduced by receptor associated Smads, Smad2/Smad3 with the co-Smad, Smad4. Upon ligand binding to the receptors, Smad2/Smad3 gets phosphorylated and translocates to the nucleus in a complex with Smad4. In the nucleus, the Smad complex bind to transcription factors FoxH1 and Mixer to activate transcription of target genes.

During zebrafish development, the roles of Nodal signaling in mesendoderm induction and patterning, neural patterning and left-right axis specification are well studied (reviewed in (Schier, 2003; Schier, 2009b; Shen, 2007). In addition to these, Sampath laboratory has discovered a non-coding function of asymmetrically localized maternal sqt transcripts in dorsal axis specification (Gore et al., 2005; Lim et al., 2012). In mature oocytes, sqt transcripts are distributed throughout the yolk in discrete puncta. Upon egg activation or fertilization, these puncta form bigger aggregates and translocate to the blastoderm by a microtubule dependent mechanism (Gore and Sampath, 2002). By the 4-cell stage, sqt RNA is asymmetrically localized to one or two cells and the cells acquiring sqt are required for formation of dorsal structures (Gore et al., 2005) (Figure 1.9). Further analysis by morpholino knockdown of sqt and overexpression of sqt 3'UTR showed that sqt localization is upstream of nuclear translocation of β catenin and the dorsalizing function lies in the sqt 3'UTR which requires Wnt/β catenin signaling (Lim et al., 2012). Nodal signaling per se is not required for initial dorsal specification during the early cleavage stages consistent with the requirement of Nodal receptors and

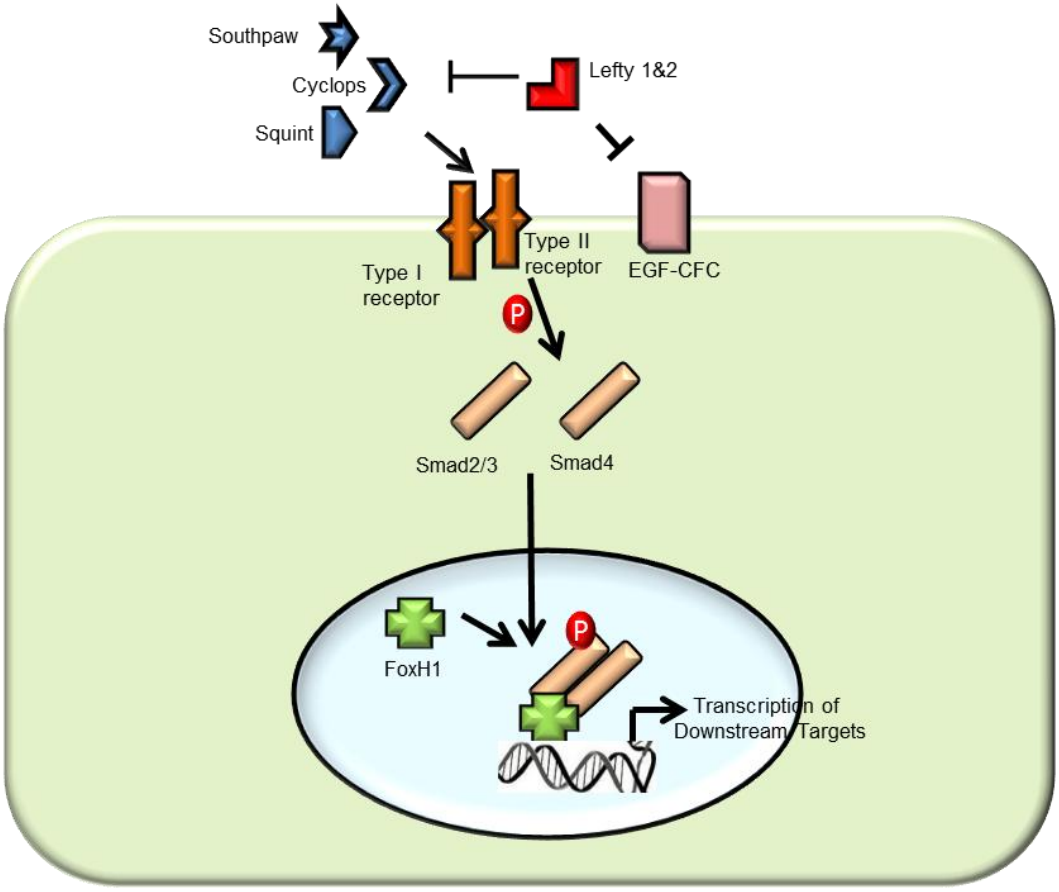


Figure 1.8 – *Schematic outline of Nodal signaling pathway.*

A simplified description of Nodal signaling pathway showing ligands (Squint, Cyclops, Southpaw) bind to Type I and Type II receptors. Co-receptor, EGF-CFC (Oep) is required for signaling. Extracellular inhibitors like Lefty proteins can regulate signaling. In response to ligand binding, Smad proteins mediate signal transduction and activate transcription factors (FoxH1, Mixer) to initiate transcription of downstream genes. Adapted from (Shen, 2007)

co-receptor, Oep, from late blastula stages (Gritsman et al., 1999; Hagos and Dougan, 2007).

1.4 Research objectives

The asymmetric localization of maternal sqt RNA is required for dorsal specification. The information required for dorsal specification lies in the sqt 3' UTR and requires Wnt signaling but not Nodal signaling. Some of the questions raised by these observations are –

How is maternal sqt regulated?

What are the *cis* elements and *trans* factors required for asymmetric localization of sqt?

Why is Nodal signaling not activated till late blastula stages despite the availability of maternal sqt RNA?

So, during my PhD work, I identified the *cis* elements and *trans* factors (sqt RNA binding proteins) required for sqt RNA localization and characterized the role of a sqt RNA binding protein in regulation of maternal sqt RNA and Sqt/Nodal signaling.

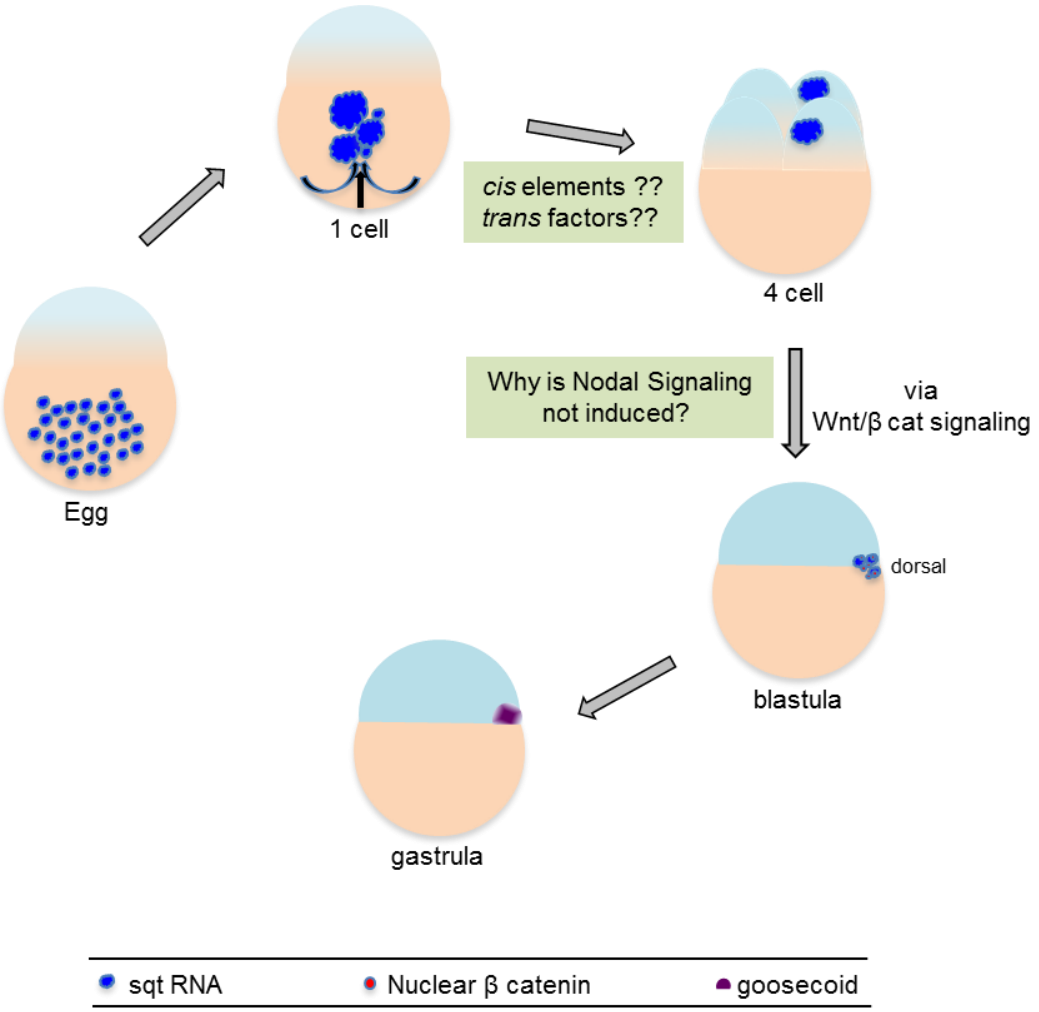


Figure 1.9 – *Schematic elucidating role of maternal squint transcripts and related questions.*

Maternally deposited sqt transcripts are seen as discrete punctate in the yolk of mature eggs. Upon egg activation/ fertilization, sqt granules aggregate and translocate to the blastoderm and asymmetrically localize by the 4-cell stage. Localization of sqt and associated factors, results in nuclear localization of β-catenin in a small cluster of cells in blastula stage embryos. Wnt signaling is required for this dorsal function of sqt RNA, while Nodal signaling is not required. Adapted from (Gore et al., 2005; Lim et al., 2012)

Chapter 2

Materials and Methods

2.1 Molecular Biology and Recombinant DNA techniques

2.1.1 Generation of Constructs

The coding sequence of *ybx1* was amplified by PCR (with primers including restriction sites, for *Nco*I and *Bam*HI or *Bgl*II) from zebrafish ovary or embryo cDNA, restriction digested, and cloned into pTrcHISA. Mutations in pCS2-sqt (Gore et al., 2005) and pTrcHISa-ybx1 plasmids were made by site-directed mutagenesis as described (Zheng et al., 2004). The template plasmids were amplified by PCR with partially overlapping forward and reverse primers harboring the mutation, (Table 2.1) using Vent Polymerase (NEB) in a 50 µl reaction

Plasmid	50ng
2mM dNTP	10 µl
Forward Primer	10 pmol
Reverse Primer	10 pmol
10X Buffer	5 µl
Vent Polymerase	1 U

PCR program was as follows –

1. 94^{o} C	5 min
2. 94^{o} C	20 sec
3. 55^{o} C	20 sec
4. 68^{o} C	8 min
5. Goto step 2	15 times
6. 72^{o} C	10 min

PCR products were digested with *Dpn*I, and transformed into XL1blue cells. Plasmids were then isolated and sequenced to identify the mutation containing plasmid.

2.1.2 Semi-quantitative and Quantitative RT-PCR

Total RNA was extracted from embryos using TRIzol reagent (Invitrogen) and purified according to manufacturer's instructions. 1 µg of total RNA from WT, P*ybx*^{sa42} or M*ybx*^{sa42} embryos was used for cDNA synthesis. Reverse transcription was performed using either oligo dT or random hexamer (dN_6) primers and SuperScriptII first-strand synthesis kit (Invitrogen) following manufacturer's instructions. cDNA was diluted 5-10 times depending on the expression level of RNAs being analyzed. As a control, reverse transcription was performed in the absence of reverse transcriptase (RT-). Genomic DNA contamination was checked by PCR with actin and squint primers. Semi-quantitative PCRs were done with number of cycles falling in the linear range of amplification in a 20 µl reaction –

cDNA	1 µl
2 mM dNTP	2 µl
Forward primer	10 pmol
Reverse primer	10 pmol
5X GoTaq buffer (Promega)	4µl
$MgCl_2$	2 mM
GoTaq G_2 Flexi Polymerase (Promega)	2 U

Real-time PCR was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using the comparative C_T method. Control experiments to measure changes in C_T with template dilutions were performed to test whether amplification efficiencies of target (*sqt, lft2, gsc, ntl, bon, gata5, boz, vox, vent, pea3, spry4, mxtx2, hhex, cldE and krt4*) and control (*act*) primers were similar. All results were normalized to *act.* 10 µl reactions were setup as following –

cDNA	1 µl
Forward and reverse primers	10 pmol
2X SYBER green master mix (Applied Biosystems)	5µl

2.1.3 Capped mRNA and DIG labeled antisense probe synthesis

All reagents used should be RNase free or DEPC treated. Chloroform and isopropanol used for RNA purification are stored in -20^{o} C. Capped mRNA was synthesized from linearized plasmids using the SP6 mMessage mMachine Kit (Ambion) in a 20 µl reaction (incubated at 37^{o}C for 3 hours) -

Linearized plasmids	1 µg
2X NTP	10 µl
10X reaction buffer	2 µl
SP6 enzyme mix	1 µl

Plasmid DNA was digested by adding 2U of Turbo DNaseI (Ambion). The reaction was stopped by adding 15 µl of Ammonium acetate stop solution. RNA was purified by chloroform extraction followed by isopropanol precipitation (1 hour in -80^{o} C or overnight in -20^{o} C). RNA concentration was measured and small aliquots were stored in -80^{o} C.

Alexa 488 labeled capped RNA were transcribed from linearized plasmids in a 50 µl reaction (incubated at 37^{o} C for 3 hours) –

Linearized plasmid	1 µg
10 mM rNTP (rATP, rCTP, rGTP)	5 µl
10 mM rUTP	1.5 µl
Chromatide Alexa 488 rUTP (1mM) (Molecular Probes, Invitrogen)	3 µl
5X Transcription buffer (Promega)	4 µl
100 mM DTT	1 µl
SP6/T3/T7 RNA polymerase (Promega)	2 µl
RNasin (Promega)	1 µl

Plasmid DNA was digested by adding 2U of Turbo DNaseI (Ambion). RNA was first purified using the Micro Bio-Spin® 30 columns (BIO-RAD) to remove any unincorporated nucleotides. Further purification was done by chloroform extraction and isopropanol precipitation (1 hour in -80^{o} C or overnight in -20^{o} C).

Antisense DIG labeled probes for *in situ* hybridization were transcribed from linearized plasmids in a 50 µl reaction (incubated at 37^{o} C for 3 hours) –

Linearized plasmid	1 µg
10X DIG labeling mix (Roche)	5 µl
5X Trasncription buffer (Promega)	8 µl
100 mM DTT	1 µl
SP6/T3/T7 RNA polymerase (Promega)	2 µl
RNasin (Promega)	1 µl

Plasmid DNA was digested by adding 2U of Turbo DNaseI (Ambion). RNA was precipitated by adding 5 µl of 7.5 mM LiCl, 1 µl of 0.5 M EDTA and equal volume of isopropanol (1 hour in -80^{o} C or overnight in -20^{o} C).

2.2 Maintenance of Zebrafish and embryo manipulation

2.2.1 Zebrafish Strains

Wild type, *ybx1*^{sa42}, *ybx1*^{sg8}, *sqt*^{cz35} and *oep*^{tz57} fish were maintained at 28.5^{o}C, and embryos were obtained by natural mating using standard procedures, in accordance with institutional animal care regulations (Westerfield, 2007).

2.2.2 Generation of *ybx1* mutant zebrafish

Libraries of ENU-mutagenized zebrafish were screened for point mutations in the coding region of *ybx1* (de Bruijn et al., 2009; Kettleborough et al., 2011; Winkler et al., 2011). Oligonucleotides were designed against exons two to four of zebrafish *ybx1* located on chromosome 8: 49299968 to 49308225 (Ensemble Zv9). This region was amplified by nested PCR using the primers listed in Table 2.1. Sanger sequencing of PCR fragments was performed with the universal M13 forward sequencing primer AGGAAACAGCTATGACCAT. Primary hits were amplified and re-sequenced independently and verified. Mutant *ybx1*^{sa42} zebrafish (which harbor a V83F amino acid substitution) were propagated further and bred to homozygosity. For generating deletions in the *ybx1*

locus we used zinc finger nuclease technology. A pair of zinc fingers recognizing exon 5 of *ybx1* were designed (Toolgen Inc.) and fused to the *Fok*I nuclease domain (Amacher, 2008; Doyon et al., 2008; Meng et al., 2008). Capped mRNA was synthesized from linearized plasmids and 25pg RNA of each zinc finger nuclease pair was injected in 1-cell stage wild-type embryos. Injected embryos were raised to adulthood and progeny were screened for mutations in the *ybx1* locus by PCR and sequencing. We identified several small deletions at the target site. The *ybx1*^{sg8} allele used in this study has a 5-nucleotide deletion in exon 5 of *ybx1*, which leads to a frame-shift after amino acid residue 197 and premature termination after amino acid residue 205.

2.2.3 Generation of *ybx1* rescue transgene

A 8.26 kb *ybx1* genomic fragment was amplified by PCR, fused with the viral peptide 2a and gfp sequences, cloned into pMDs6 plasmid and co-injected with Ac II transposase mRNA into *ybx1*^{sa42} embryos at the 1-cell stage (Emelyanov et al., 2006). Injected embryos were raised to adulthood, and progeny were screened for GFP expression. Two independent *Tg*(*ybx1-2a-gfp)* transgenic lines were used in this study.

2.2.4 Genotyping mutants

Genomic DNA from tail-fins/embryos was extracted for various mutant lines and genotypes were determined by PCR based methods using primers in Table 2.1. Tail-fins/embryos were digested in lysis buffer (100mM Tris-HCl pH8, 200mM NaCl, 1mM EDTA, 0.1%SDS and 0.2mg/ml proteinase K) by incubating at 55°C overnight. DNA was purified by phenol-chloroform extraction followed by isopropanol precipitation.

ybx1^{sa42}

Primers specific to *ybx1* (Figure 2.1A, Table 2.1) were used to amplify ~500bp of genomic locus surrounding the V83F mutation (GTT→TTT). This mutation creates a restriction site for AluI. PCR products were digested with AluI (NEB) and digested products are analysed on a 1.6% agarose gel (Figure 2.1B).

ybx1^{sg8}

Forward primers were designed to distinguish between the WT and *ybx1*^{sg8} sequences (Figure 2.1C, D, and Table 2.1). They were used with a common reverse primer to amplify a product of ~150 bp. In order to confirm we also amplified a ~300 bp region (Ybx1ScFw2 - Ybx1ScRev3) surrounding the sg8 deletion and sequenced the PCR products (Figure 2.1E).

ybx1^{sa42};sqt^{cz35}

ybx1^{sa42}*;sqt*^{cz35} double mutants were generated. Adult fishes and embryos were genotyped for *ybx1*^{sa42} mutation as described earlier. For genotyping *sqt*^{cz35} mutation forward primers were designed to distinguish between WT and mutant DNA (Figure 2.1F, G, and Table 2.1).

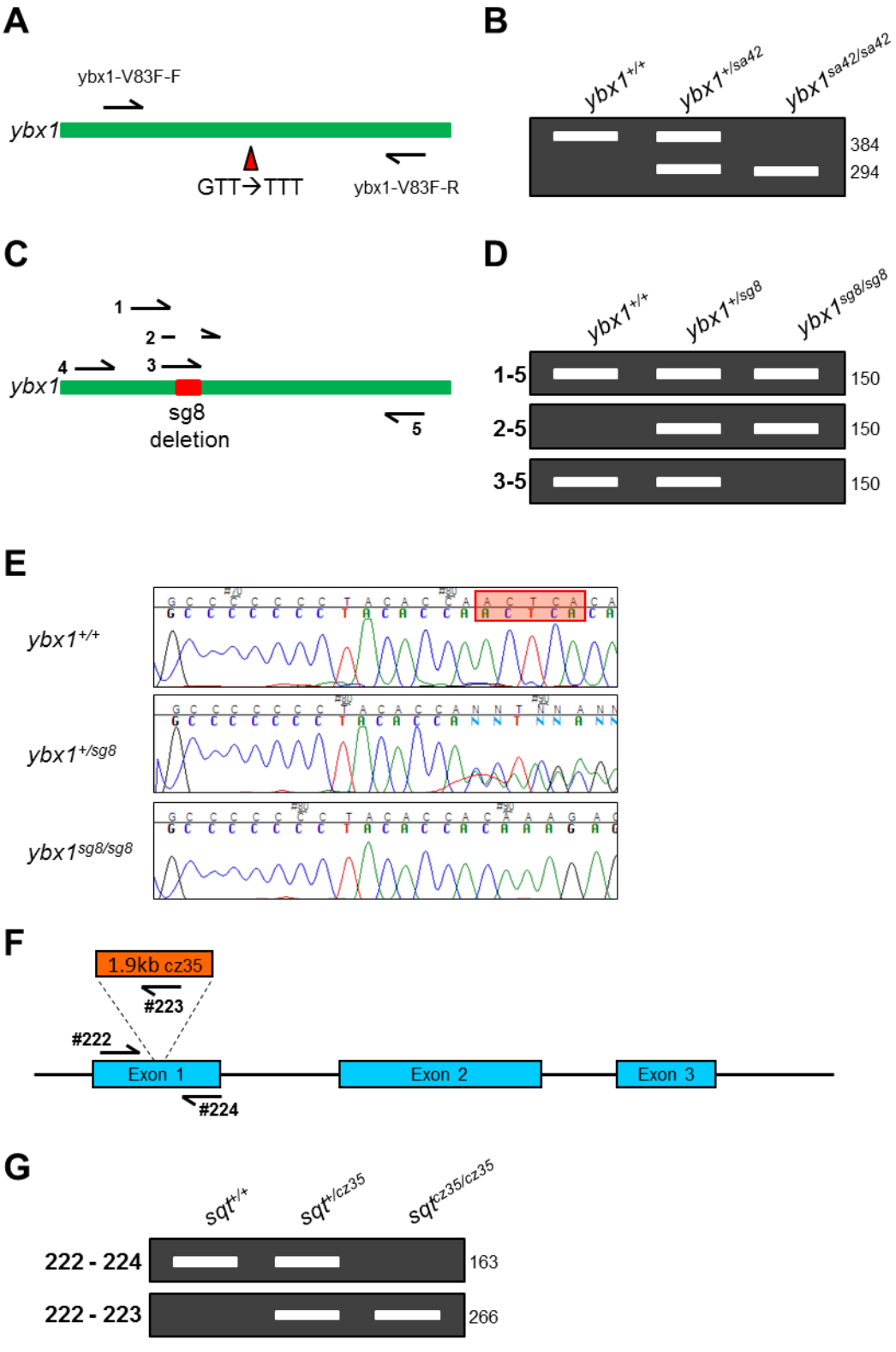

A
ybx1-V83F-F
ybx1
GTT→TTT
ybx1-V83F-R
B
ybx1+/+
ybx1+/sa42
ybx1sa42/sa42
384
294
C
1
2
3
4
ybx1
sg8
deletion
5
D
ybx1+/+
ybx1+/sg8
ybx1sg8/sg8
1-5
2-5
3-5
150
150
150
E
ybx1+/+
ybx1+/sg8
ybx1sg8/sg8
F
1.9kb cz35
#223
#222
Exon 1
Exon 2
Exon 3
#224
G
sqt+/+
sqt+/cz35
sqtcz35/cz35
222 - 224
222 - 223
163
266

Figure 2.1 – *Genotyping strategies*

(A) Schematic showing the *ybx1* gene region harboring the sa42 (GTT→TTT, V83F) mutation and positions of primers to amplify this region. (B) Schematic representation of a DNA gel showing PCR products, for wild type, heterozygous and homozygous *ybx1*^{sa42} fishes, digested with AluI. (C) Schematic showing the *ybx1* gene region harboring the sg8 (ΔACTCA) mutation and positions of primers to amplify this region. Primer pair 1-5 will amplify both WT and mutant DNA, primer pair 2-5 will amplify only mutant DNA and primer pair 3-5 will amplify only WT DNA (D) Schematic representation of a DNA gel showing PCR products, for wild type, heterozygous and homozygous *ybx1*^{sg8} fishes. (E) Chromatograms showing sequencing results for PCR products amplified by primer pair 4-5 in D. The sg8 deletion –ACTCA is marked by red box in the WT sequence. (F) Schematic showing sqt genomic locus (not drawn to scale) and site for cz35 insertion. Primer pair 222-224 will amplify WT DNA and primer pair 222-223 will amplify mutant DNA. (G) Schematic representation of a gel showing PCR products from WT, heterozygous and homozygous *sqt*^{cz35} fishes.

2.2.5 Temperature shift experiments

Embryos from mating of homozygous *ybx1*^{sa42} females were collected, incubated at 28.5^{o}C until the first cell division, and then shifted to 23^{o}C for observing the temperature-sensitive phenotype. A few homozygous *ybx1*^{sa42} females yield embryos that manifest a range of phenotypes, some of which survive at 23^{o}C. In this study, homozygous *ybx1*^{sa42} females that yielded 100% embryos arrested at gastrula stages were used in all experiments. Embryos from homozygous *ybx1* males and wild-type females (P*ybx1*), are indistinguishable from wild-type embryos, and were used as controls. For examining *ybx1;sqt* double mutant phenotypes, embryos from matings of *ybx1*$^{sa42/sa42}$*;sqt*$^{cz35/+}$ fish were incubated at 28.5^{o}C until the 4-cell stage to allow sqt RNA localization, shifted to 23^{o}C until the 128-cell stage, and subsequently returned to 28.5^{o}C until observation at gastrula and prim-5 stages. The genotypes of mutants were determined by PCR as described (Figure 2.1F, G).

2.2.6 Fluorescent capped RNA and morpholino injections

20pg aliquots of fluorescently labeled RNA were injected in 5-10 minutes post fertilization (mpf) 1-cell stage embryos. Asymmetric localization was scored at the 4-cell stage, visually by two individuals. Discrete punctate in one or two cells on one side of the embryo was scored as asymmetric localization (Gilligan et al., 2011). Live embryos were imaged at the 4-cell stage using a Zeiss Axioplan2 upright microscope and CoolSNAP Photometrics camera (Roper Scientific). For antisense morpholino oligo injections, 20 pg of fluorescent RNA was co-injected with 4 ng of the morpholinos (sequences in Table 2.2).

2.2.7 Lefty RNA injections

Capped synthetic lefty1 RNA was synthesized from linearized plasmid as described earlier. 2 pg aliquots of lefty1 RNA were injected into M*ybx1*^{sa42} mutant or P*ybx1*^{sa42} control embryos at the 1-cell stage. Capped lacZ RNA was injected as a control. The embryos were incubated at 28.5^{o}C until the 4-cell stage

to allow sqt RNA localization, shifted to 23°C until the 256-cell stage, and subsequently returned to 28.5°C until observations at gastrula and prim-5 stages.

2.2.8 Bead Implantation

Affi-Gel blue beads (50-100 mesh, Biorad) were pre-soaked in Bovine Serum Albumin (BSA; 100µg/ml; NEB) or mouse Nodal protein (125-250 µg/ml, R&D systems) for 30 minutes. Single beads were implanted into the yolk of de-chorionated 32-cell stage embryos by making a small incision in the yolk with a tungsten needle, and nudging the Affigel bead into the yolk with pair of fine forceps (von der Hardt et al., 2007). For DAPI or SYTOX staining, implanted embryos were cultured in 30% Danieau's buffer, fixed at the 1000-cell stage, and stained.

2.3 Biochemistry

2.3.1 RNA gel-shifts and UV-crosslinking assays

Extracts were made by homogenizing embryos in 1 volume of lysis buffer (20 mM TRIS pH 8.0, 100 mM NaCl, 0.1mM EDTA, 1 mM 6 aminohexanoic acid, 1 mM PMSF, 25% glycerol) on ice with a dounce homogenizer. Debris was pelleted by centrifugation at 14000 rpm, 4°C, for 10 minute, and the supernatant was flash frozen in 50 µl aliquots in liquid nitrogen. Transcription templates for probe synthesis were generated by PCR with an extended phage T3 RNA polymerase promoter (AATTAACCCTCACTAAAGGGAGAA) appended to the 5'end of the 5' primer, and gel-purified. Primers are listed in Table 2.1. Radioactively-labeled probes were transcribed in 3 µl reactions containing 0.5 µl template, 1.5 µl αP^{32} UTP (3 µM), and 0.6 µl 5X transcription buffer (Promega), 0.2 µl RNasin (Promega), 2.5 mM rATP, rGTP and rCTP, and 0.025 mM rUTP at 37°C for 3 h. The reaction was stopped by adding 40 µl TE containing 30% glycerol and ~0.01 % Bromophenol Blue. Probes were not usually denatured. 1 µl of extract (~20–50 µg of protein) was pre-incubated with 4 µl of 2X gel-shift buffer (20 mM Hepes (pH 7.9), 100 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 20

mM DTT, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM $ZnSO_4$, 60 % glycerol (or 2M betaine), 500 µg/ml heparin, 50 µg/ml torula RNA (Sigma, R6625)) plus any competitor. The reaction was made up to 7 µl with sterile water, incubated for 5 minutes at room temperature, following which 1 µl of probe (~ 1–2 ng, ~ 10^5 cpm) was added. The reaction was further incubated for 5 minutes and loaded onto the gel, electrophoresed at ~25 mA (for 1 mm thick gels) for 100 – 120 minutes, dried, and auto-radiographed. For discontinuous electrophoresis of gel-shifts, the cathode buffer was 25 mM Tricine, 2.5mM TRIS, pH 7 (or 47 mM glycine, 6 mM TRIS, 0.2 mM EDTA, pH 8), the stacking gel was 25 mM TRIS pH 6.8, 3% 39:1 acrylamide:bisacrylamide, the resolving gel was 0.5 X TBE (45 mM Tris-borate pH 8.3, 1 mM EDTA), 4 - 6% 29:1 acrylamide:bisacrylamide, and the anode buffer was 0.5X TBE. Continuous gels were 0.5X TBE, 4 - 6% 39:1 acrylamide:bisacrylamide, with a 0.5XTBE running buffer. RNA cross-linking reactions were essentially the same as RNA gel-shifts, except that the reactions were UV-cross-linked for 5 minutes in a Stratalinker (Stratagene), digested with RNase A (0.5 µg) for 1h at 37^{o}C, and separated on an SDS-PAGE gradient gel (6-20%) at ~ 25 mA for ~6 hours, dried, and auto-radiographed.

2.3.2 Chromatographic purification of proteins

Extracts were made as above, and flash frozen in 2 ml aliquots. Chromatography was performed on an Akta purifier (GE Healthcare). 200–500 mg of protein extract was injected through a 0.2 µm syringe filter ("Minisart", Sartorious) at 1 ml/minute to chromatographic columns (GE Healthcare) pre-equilibrated in 20 mM TRIS pH 8.0, 10% glycerol, and eluted with a 50-100 ml 0 to 1M $(NH_4)_2SO_4$ gradient. Fractions of 1.8 ml were collected and assayed by gel-mobility shift with sqt probes. Positive fractions were pooled, dialyzed and loaded onto the next column. We used 1-5 µl of each fraction for gel-shifts or RNA cross-linking assays. We concentrated 0.5-1 ml of each fraction to 100 µl in a 400µl spin column (Vivaspin 500), and loaded 50 µl on an SDS-PAGE gradient gel. The gel was stained with colloidal Coomassie blue (Kang et al., 2002) and

the band that co-fractionated with RNA binding activity was excised and sequenced.

2.3.3 RNA immunoprecipitation

RNA-IP was carried out using embryos lysates as described (Niranjanakumari et al., 2002). 20 mpf embryos were cross-linked (1% formaldehyde, 20 minutes), and lysed in equal volume of RIPA buffer (50 mM Tris-Cl pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, protease inhibitor cocktail). 2 µg of anti-Ybx1 (Sigma 4F12), anti-eIF4G (Cell Signaling #2469) and anti-eIF4E (Cell Signaling #2067) antibodies was bound to 50 µl of protein A/G beads (Calbiochem), incubated with 250 µl wild-type embryo lysates at 4^{o} C overnight, washed with high stringency RIPA buffer (50 mM Tris-Cl pH 7.5, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1M NaCl, 1 M Urea (optional), protease inhibitor), and eluted with 100 µl of elution buffer (50 mM Tris-Cl pH 7, 5 mM EDTA, 10 mM DTT, 1% SDS) by heating at 70^{o}C for 10 min. Half of the eluate was used to detect proteins by western blot and the remainder was used for RNA extraction using TRIzol reagent (Invitrogen), followed by cDNA synthesis (First strand synthesis kit, Invitrogen). Expression of sqt, wnt8a and gapdh was detected by PCR (primer details in Table 2.1).

2.3.4 Protein expression and detection

E. coli BL21 cells were transformed with plasmids encoding wild-type and mutant Ybx1. 2 ml cultures at OD_{600} were induced with 0.25 mM IPTG for 12 h at 28^{o}C, pelleted, and lysed in 200 µl lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8), by vortexing with glass beads. Debris was pelleted (20,000 g, 4^{o}C, 2 minutes), and aliquots of supernatants were flash frozen. Expression of recombinant protein was detected by western blots with an anti-6xHis antibody (1:2500 Santa Cruz Biotechnology Inc., sc50973), and equal amounts of *E coli* lysates were used in gel-shift assays.

To detect Sqt translation, P*ybx1*^{sa42} and M*ybx1*^{sa42} embryos were injected with 20 pg sqt-GFP RNA. Whole embryo lysates (50µg) were separated on an 8% SDS-PAGE gel, transferred to High bond-C Extra Membrane (GE Healthcare), and immunoblotting was performed using anti-GFP primary antibodies (1:2500, Abcam ab290) and HRP-conjugated anti-rabbit IgG secondary antibodies (1:10000, DAKO). Endogenous phospho-Smad2 was detected using anti-PSmad2 primary antibodies (1:1000, Cell Signaling #3101), and HRP-conjugated anti-rabbit IgG secondary antibodies (1:5000, DAKO). Endogenous Ybx1 expression in embryos was detected using a mouse anti-Ybx1 antibody (1:1000, Sigma 4F12), and HRP-conjugated anti-mouse IgG secondary antibody (1:10000, DAKO). Anti-eIF4E (1:2000, Cell Signaling #2067) and anti-eIF4G (1:2000, Cell Signaling #2469) antibodies were used in co-immunoprecipitation assays and western blots to detect interactions with Ybx1.

2.4 Staining and Imaging techniques

2.4.1 RNA in-situ hybridization

Fixed embryos were processed for whole mount *in situ* hybridization using digoxygenin (DIG) labeled anti-sense RNA probes to detect *claudinE, cyclinb, eomesodermin, goosecoid, mxtx2, squint, vasa, vox, wnt8a,* and *ybx1* expression (Du et al., 2012; Gore et al., 2005; Hong et al., 2011; Howley and Ho, 2000; Lim et al., 2012; Lu et al., 2011; Melby et al., 2000; Siddiqui et al., 2010; Stachel et al., 1993; Yoon et al., 1997).

Embryo Preparation

1-cell and 4-cell stage embryos were fixed in fish fix buffer containing 4% paraformaldehyde, 4% sucrose, and 120 µM calcium chloride in 0.1M Phosphate buffer (pH 7.2). Blastula, gastrula and prim5 stage embryos were fixed in 4% paraformaldehyde/PBS (PFA) overnight at 4^{o} C. Embryos were washed 3 times with PBST (0.1% Tween 20 in PBS) and dechorionated using forceps. Embryos were dehydrated by using a gradient of methanol (25%, 50% and 75% in PBST) and stored in 100% methanol in -20^{o} C.

Hybridization

Embryos were rehydrated using a gradient of methanol (75%, 50% and 25% in PBST) and washed with PBST for 3 times. After proteinase K digestion (3µg/ml) for 1-5 minutes, embryos were post-fixed with 4% PFA for 20 minutes and washed with PBST 3 times. Embryos were then pre-hybridized for 4 hours at 65^{o} C in the hybridization buffer (60% Formamide, 5X SSC, 1mg/ml torula RNA, 100µg/ml heparin, 1X Denhardt's solution, 0.1% CHAPS, 10mM EDTA, 0.1% Tween-20, adjust pH to 6.0-6.5 with 1M citric acid.). DIG labeled probes (1-5 ng/µl) were added to the hybridization buffer and embryos were incubated at 65^{o} C overnight.

The non-hybridized probe was washed as follows at 65^{o} C –

10 mins	100% FSTw (60% Formamide, 5X SSC, 0.1% Tween 20)
10 mins	75% FSTw/ 25% 2X SSCTw.
10 mins	50% FSTw/ 50% 2X SSCTw
10 mins	25% FSTw/ 75% 2X SSCTw
3X 10 mins	2X SSCTw.
2X 30 mins	0.2X SSCTw

Following washes are done at room temperature.

5 mins	75% 0.2X SSCTw/ 25% MABTw
5 mins	50% 0.2X SSCTw/ 50% MABTw
5 mins	25% 0.2X SSCTw/ 75% MABTw
2X 5 mins	MABTw

Antibody binding

Embryos were blocked with 1% Roche Blocking Reagent in MABTw for 2h at room and then incubated in 1:2000 dilution of pre-adsorbed anti-DIG-alkaline phosphatase antibody made in 1% Roche Blocking Reagent in MABTw, for 4h at room temperature (RT) or overnight at 4^{o} C. The unbound antibody was washed away by 8X 15 mins washes with MABTw.

Detection

Embryos were equilibrated in freshly prepared NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 1% Tween 20, 1mM Levamisole) by 3, 10mins washes. Staining was developed in dark by adding alkaline phosphatase substrate, BM Purple (Roche). Staining was stopped by several washes with PBST followed by fixing with PFA for 20 mins. Stained embryos are stored in 50% glycerol/ 50% PBS at 4^o C.

2.4.2 Membrane and nuclear staining

We used anti-E-cadherin antibodies to detect cell membrane adhesions. Control or mutant embryos at the 1000-cell stage were fixe in 4% paraformaldehyde/PBS and processed for fluorescence immunohistochemistry using rabbit polyclonal anti-E-cadherin antibodies (gift from CP Heisenberg) and Alexa-488-conjugated goat anti-rabbit secondary antibodies (Molecular Probes). Fixed embryos were washed with PBST and manually dechorinated. Embryos were permeabilized by dehydrating in a gradient of methanol in PBST (25%, 50%, 75% and 100%). After rehydrating back to PBST, embryos were incubated in blocking solution (1% DMSO, 1% BSA in PBST) for 2-4 hours at room temperature and then in primary antibody (1:200 in blocking solution) for overnight at 4^o C. Unbound antibody was washed with 6, 20 mins washes with PBST. Embryos were then incubated with secondary antibody (1:1000 in blocking solution) for overnight at 4^o C. Unbound antibody was washed with 6, 20 mins washes with PBST and stained embryos were stored in 4^o C before imaging.

For detecting nuclei, embryos were fixed with 4% paraformaldehyde/PBS, washed with PBST, incubated with 500 pg/ml DAPI, and washed with PBST. To label yolk syncytial nuclei in live embryos, 4 nl of 0.5 mM SYTOX orange (Invitrogen) was injected into yolk of 64-cell stage embryos. Labeled nuclei were scored at 512-1K cell stages.

2.4.3 Microscopy

Live embryos, for DIC or fluorescence (injected with fluorescent RNAs or expressing GFP protein) imaging, were manually dechorionated, mounted in 2.5% methylcellulose (Sigma) and imaged using Zeiss Axioplan2 microscope with a CoolSNAP HQ camera (Photometrics). MetaMorph (Universal Imaging Corporation) and ImageJ (NIH) software packages were used to acquire and process images respectively. Stained embryos from in *situ* hybridization experiments were mounted in 100% glycerol and imaged using a Zeiss Axioplan2 microscope equipped with a Nikon DXM1200 color camera. Images were acquired using ACT-1 software (Nikon) and processed using ImageJ (NIH). For E-cadherin and DAPI-stained embryos, images were acquired using a Zeiss LSM 5 Exciter upright confocal microscope or Zeiss LSM 510 META inverted microscope and maximum intensity projections were made and processed using ImageJ (NIH).

Table 2.1 Primer Sequences

Primer Name	Sequence
For Templates to transcribe gel-shift probes:	
sqt.1T3-F	AATTAACCCTCACTAAAGGGAGATCATGAGACACCATGAAG
sqt.1-R	AAGGAGCATATCCAAAGTGC
sqt.2T3-F	AATTAACCCTCACTAAAGGGAGATTCTTCAAACCCCAAAG
sqt.2-R	AAGTGGGAATAATTGACAGC
sqt.3T3-F	AATTAACCCTCACTAAAGGGGACCCCAAAAATATGTAT
sqt.3-R	ATAGCATCAAGTTATCCAG
sqt.4T3-F	AATTAACCCTCACTAAAGGGAGAGAAATTATTATGGTTTC
sqt.4-R	CAGATAAGGCAAACACG
sqt.5T3-F	AATTAACCCTCACTAAAGGGAGATATTGAAAGCTTTGCGT
sqt.5-R	ATTATGAAAACATTTTATTAC
MmGAPDHT3-F	ATTAACCCTCACTAAAGGGAGATGAGAAACCCTGGACCACCCAC
MmGAPDH-R	CAGTGATGGGGGCTGAGTTG
gapdhT3-F	AATTAACCCTCACTAAAGGGAGAAAAGCCAGACCATTCCTTC
gapdh-R	TTTTTAAACTGCATTACAGTAGCCTTT
cycT3-F	AATTAACCCTCACTAAAGGGAGAAGTGCGGATGCCTGTGA
cycT3-R	TGAGTGTGTGTTTGTGCGTC
wnt8a.1T3-F	AATTAACCCTCACTAAAGGGAGACCGGCCGCACAACCATTCAC
wnt8a.1-R	TATTTACATTAGAAATATAC
wnt8a.2T3-F	AATTAACCCTCACTAAAGGGAGATATTTTATGAGATTTTAAGA
wnt8a.2-R	GTGGGAACGAGAAAGCCCAT
wnt8a.3T3-F	AATTAACCCTCACTAAAGGGAGATGTCAATTGAATTCATTGAA
wnt8a.3-R	ACATTTTTTGAGAGCAACAA
wnt8a.4T3-F	AATTAACCCTCACTAAAGGGAGATTGTATTTTTTCATGCACAG
wnt8a.4-R	AAAATATTTGCCTTAAATA
vg1.1T3-F	AATTAACCCTCACTAAAGGGAGAAGTGGATGCAGATGAACATG
vg1.1-R	AAAAGAAGCCTAATTTTGC
vg1.2T3-F	AATTAACCCTCACTAAAGGGAGAACAATTTTTCTTTTTTTAGGTG
vg1.2-R	ATTATAAAAAGTTACTTTAACAGC
vg1.3T3-F	AATTAACCCTCACTAAAGGGAGAGATGCAGAGAATGTGC
vg1.3-R	GAAAAAAAAGGAATCCCATAGTAAAAG
SU-nodal T3F	AATTAACCCTCACTAAAGGGAGATAGTTCGTCACCATGAGAAC
SU-nodal-R	ATTGTAAAAGTTCAAAGTTC
Mouse-nodal T3F	AATTAACCCTCACTAAAGGGAGATCCTGGAACACCACAAGGAC
Mouse-nodal R	TCATCAGCATTGTGGAATGCAAG
Human-nodal T3F	AATTAACCCTCACTAAAGGGAGATCCTAGATCACCATAAAGAC
Human-nodal R	TTCCCAGCCTTCCAGAGTGC
For cloning Ybx1 and site-directed mutagenesis to generateYbx1 mutants:	
Ybx1-F	AAACACCATGGGCAGCGAGGCCGAGACACAACA
Ybx1-R	TGTTTAAGCTCGAGTAATCTGCTCCGCCCTGTTC
Ybx1-V83F-F	GAACAACCCCAGGAAATATCTCCGTAGCTTTGGGGACGGAGAG
Ybx1-V83F-R	CACGTCGAACTCCACAGTCTCTCCGTCCCCAAAGCTACGGAG
Ybx1-sg8-F	TACACCAACTCACAAAGAGGAGAGATGACAGGATCCAGATCTCAT
Ybx1-sg8-R	GTGATGGTGATGGTGATGAGATCTGGATCCTGTCATCTCTCC
Ybx1dssDBD-F	ATGGGCAGCGAGGCCGAGACACAAAGGAATGACACAAAG
Ybx1dssDBD-R	GCACAAAGACATCTTCCTTTGTGTCATTCCTTTGTGTCTCG
Ybx1dRNP-F	GTTTTGGGGACAGTGAAATGGTTCAATGTAAGGCAGACCGCCA
Ybx1dRNP-R	GAGATATTTCCTGGGGTTGTTCTTTTTAATGGCGGTCTGCCTTACATTG

Ybx1dCSD2-F	GGAATGACACAAAGGAAGATGTCTTTGTGCACGTTACCGGCC
Ybx1dCSD2-R	CTACCCTGCACAGGAACGCCACCCGGGCCGGTAACGTGCACAAAG
Ybx1dDIMER-F	GCCCGGGTGGCGTTCCTGTGCAGGGTAGTAAGTATAGCGACCCTG
Ybx1dDIMER-R	CTCTCTCTTCTCCCGGGGCTCTGCCTCAGGGTCGCTATACTTACTAC
Ybx1dCterm-F	CCACCTCGCGACTACCAGGAGAACTATCAGGGATCCAGATCTCATC
Ybx1dCterm-R	GTGATGGTGATGGTGATGAGATCTGGATCCCTGATAGTTCTC
Ybx1de8-F	GAACTACTACAGAGGCTTCCGACCAGGATCCAGATCTCATC
Ybx1de8-R	GTGATGGTGATGGTGATGAGATCTGGATCCTGGTCGGAAGC
Primers for sequencing ENU mutants	
forward outer primer	CAGGGATGGTAACTTTGCTC
reverse outer primer	AGGATTGAGTTTGACATCTGTG
forward inner primer with M13 forward tail	TGTAAAACGACGGCCAGT TCGGTGTAACCTGACTCTTG
reverse inner primer with M13 reverse tail	AGGAAACAGCTATGACCAT GCCTAATATTTCTAACTGTGTGGTG
RT-PCR and Q-PCR Primers:	
actinF	GGCTACAGCTTCACCACCA
actinR	TGCTGATCCACATCTGCTG
sqtA	GAACCACAGAACTGATGATA
sqtB	GCATGGTTTGTTGGAGTGAA
sqtC	TGCCGAGCACTCCAAGTATG
sqtD	CATCAAGTTATCCAGGTGCC
sqtE	CCGCTGTATATGATGCACCTC
sqtF	ATCCACCTCCAACTCAGACC
sqtG	GAGGAGAAAAACAATATATTC
sqtH	AGTCAGTCTGGCAGGAGGAA
wnt8aF	AGTAATCCTCTTTGCAAATATGTAAAG
wnt8aR	AACCTCATCGTGAAACACTGC
gapdhF	GTTCATCCATCTTTGACGCTGGTGCTG
gapdhR	GAGGCCATGTGTGCCATCAGGTCA
gsc-F	TGGAAGGATAGGCTACAACAACTAC
gsc-R	GGTATTTCGTTTCTTGAAAAAGGTT
ntl-F	TATTGCAGTCACAGCATATCAGAAT
ntl-R	AAGCTGGAGTATCTCTCACAGTACG
gata5-F	ACTAGTACGACAACACTGTGGAGGA
gata5-R	TTTTATTGTAGAGGCGTTTTCTGAC
bon-F	GAGAACTTACAAAGAACCTCAACATTTAC
bon-R	ACACTCAGGTGATCAGTTTTGATG
lft2-F	TTCATTACTGGTCTAAATCCCAAAA
lft2-R	CTCTGTCCATATCCATAGAAACCAC
bozF	GGCACTTGAGAAAGCTGGAC
bozR	GTAGTCGGTAACCGCGAAGA
voxF	GACCTCCGACATCATACGACAAG
voxR	CAGCGTCGTGTCCATCTTCG
ventF	GATACCCAGCAAGTTCTCAGTG

ventR	CTATCTTCCTCCTCTGAGTT
pea3-F	AAACCAACAGTGGGAACTCG
pea3-R	GGCTCCTGTTTGACCATCAT
spry4-F	CGGATAGACGTCCGCTTTTA
spry4-R	GGGGTGTCGATGTAGTCGTT
mxtx2-F	TCTGATCTGCAAGCAACACC
mxtx2-R	TGTCCCAAAATGCAGAATCA
hhex-F	ACCATCGAGCTGGAGAAGAA
hhex-R	GTCCTCCGCTTCCCTTTTAC
cldE-F	AGAGATTTCTACAATCCTCTGCTCA
cldE-R	GCTGGGAGTATTTCATGTTGTATTT
krt4-F	CAGGAGCTCATGAACGTCAA
krt4-R	GATCCAGAACCGAATCCTGA
ybx1-F	GAGGGGGAGATGCAGCAGC
ybx1-R	TCTGCCTCATTGGTTTGTTG
Primer for genotyping	
#222	GAGCTTTATTTCAATAACTGCGTG
#223	ATATAAAATCAGTACAACCGCCCG
#224	GCCAGCTGCTCGCATTTTATTCC
ybx1V83F-F	TTGGGGACAGTGAAATGGTT
ybx1V83F-R	GAGTCAAACTAAGCTACGACTAAAAGC
ybx1sg8-1	GGCAGACGCCCCCCCTACACCA
ybx1sg8-2	ACGCCCCCCCTACACCACAA
ybx1sg8-3	ACGCCCCCCCTACACCAACTCA
ybx1sg8-4	GAGGGGGAGATGCAGCAGC
ybx1sg8-5	TCTGCCTCATTGGTTTGTTG

Table 2.2 Morpholino Sequences

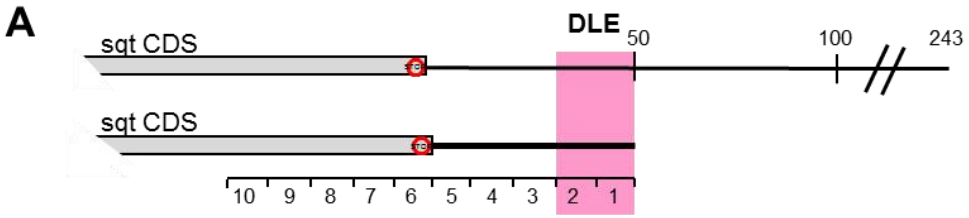
Morpholino	Sequence
sqt MO1	CAGGAGCCCGCAGGAAAACATGTCA
Con MO	CAGGATCCTGCACGAAAACGTGTCA
DLE MO	AAGGAGCATATCAAAGTGC
TP^{con} MO	TTCTTAAATACATATTTTTGGGGTC
lacZ-ATG MO	TTGGAGCAGTCATTTTTTCTGAGCT
lacZ-ATG mm MO	TTGCACCAGTGATTTTTTGTCAGCT

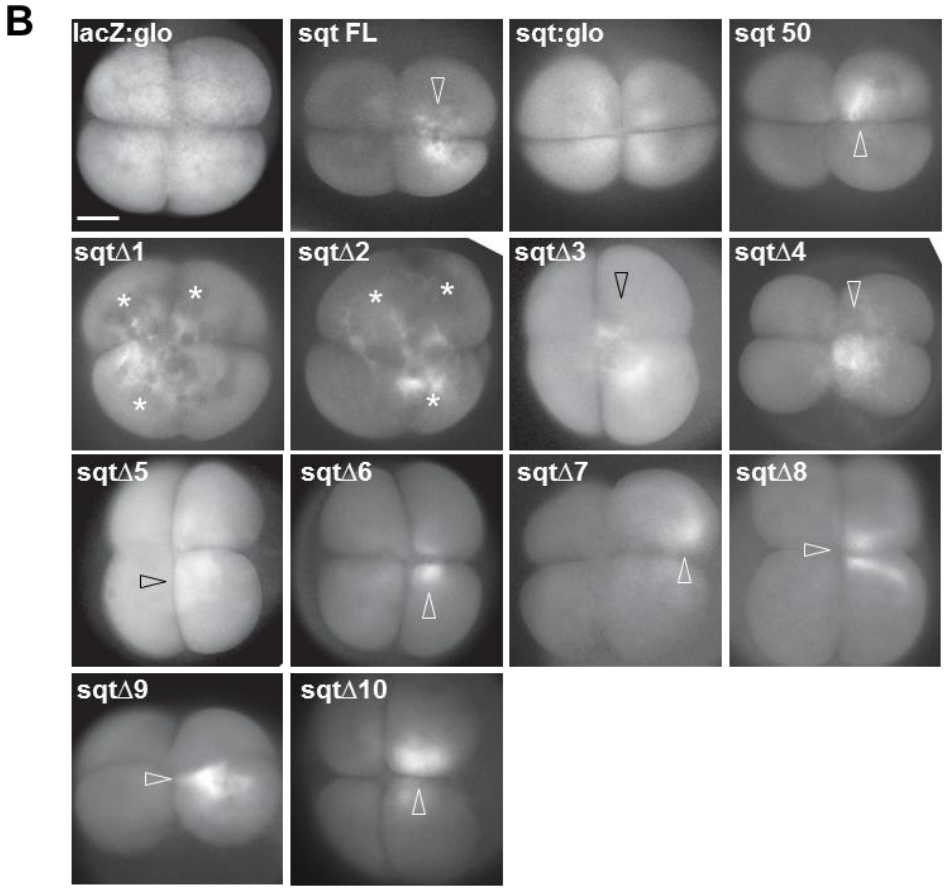
Chapter 3

Results

3.1 Mapping of sqt Dorsal Localization Element (DLE)

Maternal sqt transcripts localize asymmetrically to future embryonic dorsal by the 4-cell stage during zebrafish development, and the sqt 3'untranslated region (UTR) is necessary and sufficient to confer localization to heterologous sequences (Gore et al., 2005). By making systematic deletions within the 3' UTR, the Dorsal Localization Element (DLE) was mapped to the first 50 nucleotides in the 3' UTR (Gore et al., 2005). To map the element precisely we made further deletions in the 100 nucleotide long stretch of sqt RNA consisting of 50 nucleotides of the coding sequence and 50 nucleotides of the 3'UTR (Figure 3.1A). A series of 10 nucleotide deletions (sqt Δ1 – sqt Δ10) were tested for localization by fluorescent RNA injections in the 1-cell stage embryos and visual scoring for asymmetric distribution at the 4-cell stage (Figure 3.1B). LacZ coding sequences fused to globin 3'UTR (lacZ:glo) were used as a negative control and full-length sqt (sqt FL) or sqt open reading frame (ORF) with 50 nucleotides of the 3'UTR (sqt 50) were used as positive controls for localization. . Deletions 3-10 did not affect localization and these RNAs localized with efficiency similar to the control sqt 50 RNA. In contrast, deletions 1 and 2 affected the efficiency of localization to varying extents (Figure 3.1C). Deletion 2 reduced the percentage of embryos showing localized RNA most severely (~28%, n=125) as compared to sqt50 (~70%, n=95). We named the region 1-2 of sqt 3' UTR as Dorsal Localization Element (DLE) (Gilligan et al., 2011). RNAs with deletions in the DLE showed a patchy distribution pattern in the cytoplasm and did not localize asymmetrically (asterisks in Figure 3.1B). It is possible these deletions result in lower affinity of the RNA to the localization machinery or that certain components of the localization machinery fail to bind in the absence of the DLE.


A
sqt CDS
DLE
50
100
243
sqt CDS
10 9 8 7 6 5 4 3 2 1


B
lacZ:glo
sqt FL
sqt:glo
sqt 50
sqtΔ1
sqtΔ2
sqtΔ3
sqtΔ4
sqtΔ5
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sqtΔ8
sqtΔ9
sqtΔ10

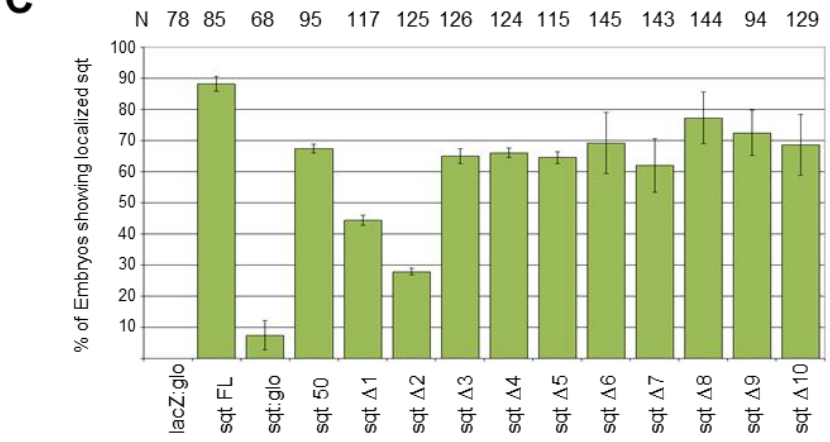

C
N 78 85 68 95 117 125 126 124 115 145 143 144 94 129
% of Embryos showing localized sqt
100
90
80
70
60
50
40
30
20
10
lacZ:glo
sqt FL
sqt:glo
sqt 50
sqt Δ1
sqt Δ2
sqt Δ3
sqt Δ4
sqt Δ5
sqt Δ6
sqt Δ7
sqt Δ8
sqt Δ9
sqt Δ10

Figure 3.1 - *Deletion analysis identifies sqt Dorsal Localization Element (DLE).*

(A) Schematic depicting sqt RNA with full length 3'UTR (sqt FL), the minimal localizing region of sqt RNA with 50 bases of 3'UTR (sqt 50) and deletion regions. (B) Fluorescently labeled RNAs – control lacZ RNA fused with globin UTR (lacZ:glo), sqt FL, sqt 50, sqt coding sequence fused to globin UTR (sqt:glo) and deletion mutants sqtΔ1 sqtΔ10 shown in (A), were injected at the 1 cell stage, and imaged from the animal pole at the 4 cell stage to score for asymmetric localization. The negative control RNA, lacZ:globin, is uniformly distributed in the cytoplasm, and the minimal localizing RNA sqt50 is asymmetrically localized (open arrowheads). In contrast, sqtΔ2 and sqtΔ1 are frequently seen in ectopic 'stringy' structures in the cytoplasm (asterisks) which are not asymmetric in distribution. (C) Graph showing frequency of localization of the RNAs. Scale bar, 100 μm.

3.1.1 sqt DLE consists of both sequence and structure

The information in *cis*-elements of RNA can be either sequence or structure or both. So, we used the RNA folding algorithm Alifold (http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi) to predict the secondary structure of the sqt UTR. RNAalifold uses sequence alignment to predict conserved secondary structures. An alignment of closely related sequences was submitted to Alifold for structure prediction (Gilligan et al., 2011). The DLE sequences are predicted to fold to a single stranded motif (AGCAC) followed by a short stem-loop (SL) (region 1-2 in Figure 3.2A). To test these structural elements we deleted the AGCAC and SL elements individually as well as together. Deletion of AGCAC (sqt ΔGCAC) and SL (sqt ΔSL) reduces the localization severely and mildly respectively (Figure 3.2B, D). Remarkably, a combined deletion (sqt ΔGCAC/SL) further reduces the localization frequency to 15 % (n=188), but does not abolish it completely, suggesting that some elements in the coding sequence might contribute to localization. In contrast, sqt Δ27 lacking all 3'UTR sequences except the AGCAC and SL regions localizes with efficiency similar to sqt 50 (Figure 3.2B, D). Point mutations in the AGCAC motif, including modifications to "UUCAC", a vegetal localization element, strongly reduced localization. Mutational analysis supports the hairpin structure prediction, as mutations that disrupt the stem reduce localization efficiency, whereas compensatory mutations that restore the stem also restore localization to levels comparable to sqt 50 (Figure 3.3C, D). These results show that the AGCAC and SL regions are the functional elements of the DLE. Hence, the DLE of sqt RNA resides in the first 50 nucleotides of the sqt 3'UTR, and encompasses both sequence and structural elements (Gilligan et al., 2011).

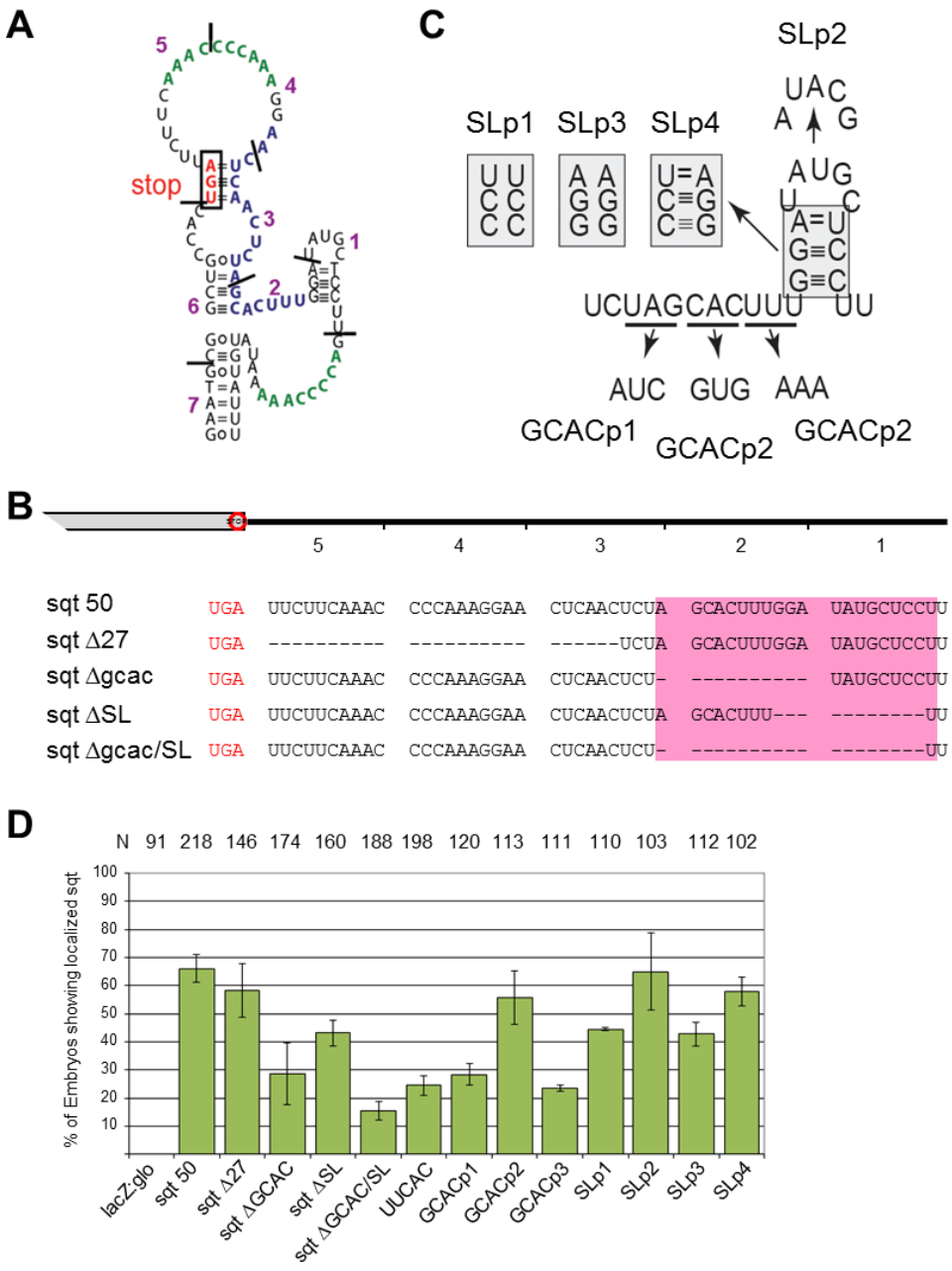


Figure 3.2 - *Mutagenesis defines localization motifs.*

(A) A predicted structure of the DLE region, showing the position of the deletions. Regions 1-2 form a stem loop. (B) Sequence of deletions to disrupt the predicted structure. DLE sequences are shaded in pink. (C) Schematic representation of the predicted single stranded AGCAC motif and the hairpin and mutations disrupting these motifs. Nucleotides forming the stem are shaded in grey. (D) Graph showing frequency of localization of deletion and point mutant RNAs.

3.1.2 ATG morpholinos against sqt 5'UTR also block localization

Dorsal activity of *sqt* is mediated by a non-coding function of maternal sqt RNA (Lim et al., 2012). However, translation-blocking morpholinos targeting sqt ATG region (sqt MO1) led to loss of dorsal structures (Gore et al., 2005). So, in order to see if sqt MO1 affects any other aspect of sqt RNA function I tested the stability and localization of sqt RNA in MO-injected embryos. Quantitative realtime PCR assays (qPCR) show that MO injections do not affect the stability of sqt RNA (Figure 3.3A, B, (Lim et al., 2012)). Next I tested if sqt MO1 affects localization. Co-injection of sqt MO1 with fluorescently labeled sqt RNA severely affects localization (90% mis-localized, n=127) and interestingly ~35% of the embryos show sqt RNA forming aggregates in the yolk that do not translocate to the blastoderm (Figure 3.3C, D). By contrast, a control MO (ATG mismatch, con MO) does not affect localization. As expected, a MO targeting the DLE also reduces localization to 40% (n=144) as compared to another MO (TP^{con}) targeting a region downstream of the DLE in the sqt 3'UTR. Therefore, in addition to blocking translation, sqt MO1 also affects sqt RNA localization.

These results point towards a possible interaction between the DLE and ATG regions of sqt RNA. Previously, it was reported that heterologous sequences such as lacZ or GFP RNA when fused to the sqt 3'UTR, localized in a manner similar to sqt (Gore et al., 2005). Therefore, to test whether sqt localization requires the sqt ATG sequences or any ATG region is sufficient to confer localization, I performed similar experiments with the lacZ ORF fused to the sqt 3'UTR (lacZ:sqt) and lacZ ATG MO (Figure 3.3A). I found that lacZ:sqt RNA localizes asymmetrically, (~50%, n=45) albeit not as efficiently as sqt:sqt (Figure 3.3C, E). Co-injection of lacZ ATG MO reduced localization of lacZ:sqt RNA dramatically to 10% (n=71) which suggests that sqt 3'UTR mediated localization of a heterologous RNA requires an ATG sequence.

Therefore, I propose that sqt RNA exists in a circularized form in the localizing ribonucleoprotein complex (RNP), and proteins that bind to sqt DLE form a complex with the ATG region of the RNA (model in Figure 3.3 F).

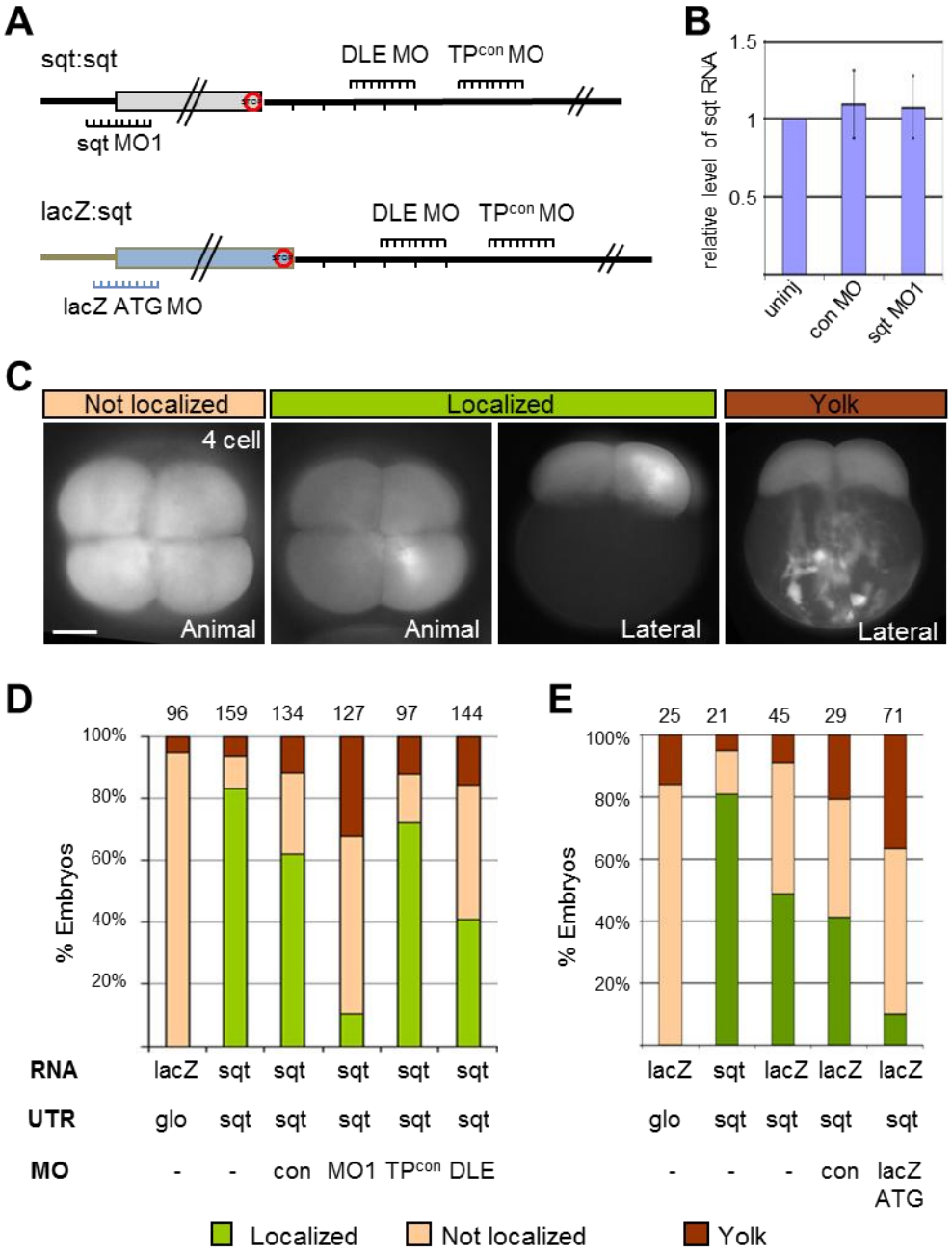

A
sqt:sqt
DLE MO
TPcon MO
sqt MO1
lacZ:sqt
DLE MO
TPcon MO
lacZ ATG MO
B
relative level of sqt RNA
1.5
1
0.5
uninj
con MO
sqt MO1
C
Not localized
Localized
Yolk
4 cell
Animal
Animal
Lateral
Lateral
D
96 159 134 127 97 144
100%
80%
60%
40%
20%
% Embryos
RNA lacZ sqt sqt sqt sqt sqt
UTR glo sqt sqt sqt sqt sqt
MO - - con MO1 TPcon DLE
E
25 21 45 29 71
% Embryos
RNA lacZ sqt lacZ lacZ lacZ
UTR glo sqt sqt sqt sqt
MO - - - con lacZ ATG
Localized
Not localized
Yolk
F
DLE
3'
sqt CDS
5'

Figure 3.3 - *Morpholinos targeting the sqt ATG and DLE regions disrupt sqt RNA localization.*

(A) Schematic of the sqt and lacZ ORF fused to sqt3'UTR (sqt:sqt, lacZ:sqt respectively) indicating positions of sqt ATG morpholino (MO1), sqt DLE morpholino (DLE MO), lacZ ATG MO and target protector control morpholino (TP^{con} MO). Stop codon is represented by a red octagon. (B) Histogram showing relative levels of sqt RNA in embryos injected with ATG mismatch MO (con MO) and sqt MO1 as compared to uninjected embryos. MO injections do not affect sqt RNA levels. (C) Animal pole and lateral views at 4-cell stage showing localization of injected fluorescent lacZ or sqt RNA in embryos co-injected with con MO, sqt MO1, TP^{con} MO or DLE MO (Green box – localized, orange box – not localized and brown box – aggregates in yolk) Scale bar, 100 µm. (D) Histogram in graph shows percentage of embryos, showing sqt RNA localized (green), not localized (orange), or as aggregates in the yolk (brown), when sqt RNA is co-injected with various MOs. (E) Histogram in graph shows percentage of embryos, showing RNA localized (green), not localized (orange), or as aggregates in the yolk (brown), when lacZ:sqt RNA is co-injected with various MOs. (F) Schematic depicting possible interaction between the DLE and ATG regions of sqt RNA mediated by a complex of proteins (colored shapes). Black lines represent UTRs, grey boxes represent exons and grey lines represent introns. DLE is marked by pink stem loop structure on 3'UTR.

3.2 Identification and purification of DLE binding factors

3.2.1 Several factors bind to sqt 3'UTR

sqt RNA localizes asymmetrically via the microtubule cytoskeleton and the *cis*-elements lie within the UTRs of sqt RNA. In order to identify the *trans*-binding factors we used a biochemical approach of RNA gel-shift assays (Electrophoretic Mobility Shift Assay – EMSA). To identify biochemical activities that recognize the sqt 3' UTR and specifically the DLE sequences, a series of overlapping 100-nucleotide long radioactive probes (Figure 3.4A) spanning the sqt 3'UTR were used for RNA gel shift with zebrafish embryo extracts. We observed a number of activities in gel shift assays with the various probes (Figure 3.4B). We named these as sqt RNA Binding Factors - SRBFs. There are at least 4 specific activities binding to specific regions of the sqt 3'UTR as shown in the schematic in Figure 3.4C.

3.2.2 SRBF1 specifically binds to sqt DLE

The DLE-containing sqt1 probe was bound by one detectable activity, SRBF1, in these assays (1 in Figure 3.4B). To determine the specificity of SRBF1 binding to the sqt DLE, we performed competition assays with zebrafish cyclops (cyc), vg1, and Green Fluorescent Protein (GFP) RNA. Zebrafish embryo extracts were incubated first with various competitor RNAs (5-80 ng), and then radioactively labeled sqt1 (~0.1 ng) was added. The sqt 3'UTR with 50 nucleotides of coding sequences competes most strongly with sqt1 (Figure 3.5A), showing that SRBF1 preferentially binds the DLE-sequences.

To precisely map the SRBF1 binding site within sqt1, a series of 10 nucleotide deletions were generated and tested for binding. Deletions in the coding sequence did not affect SRBF1 binding, whereas deletions 1– 4 (Δ1–Δ4, Figure 3.5B, C) abolish or significantly reduce binding to the sqt1 probe. Remarkably, the SRBF1 binding site overlaps with sequences previously shown to be required for dorsal localization of sqt RNA (Figure 3.5C). Thus, SRBF1 is the activity that binds to the sqt DLE.

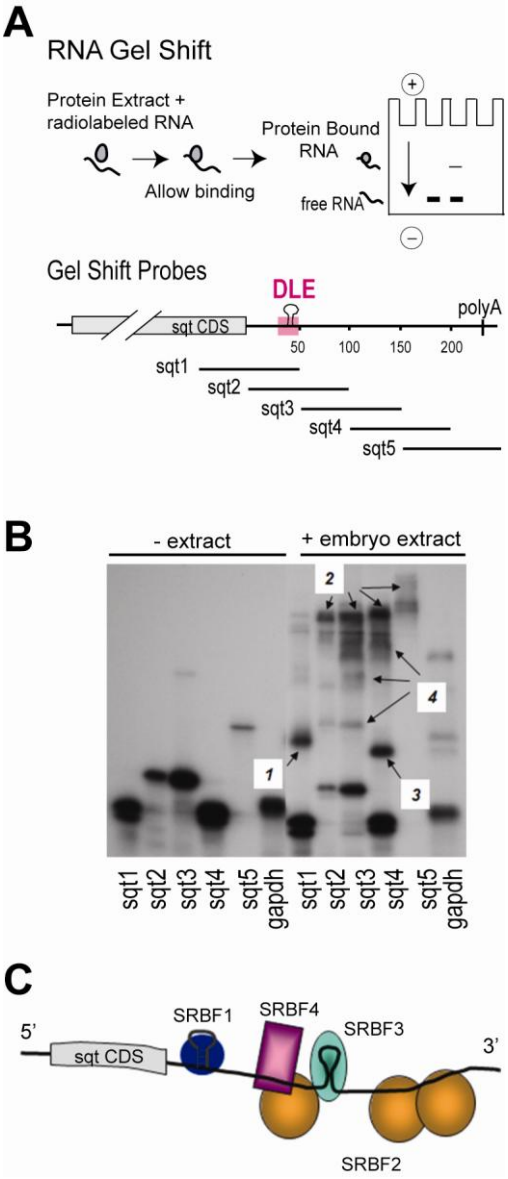


Figure 3.4 – *sqt 3'UTR is rich in RNA binding sites*

(A) Schematic elucidating the technique of RNA gel shifts and probes used. RNA gel shifts were performed with overlapping 100 nucleotide radioactive RNA probes spanning the sqt 3'UTR and extracts from 20 mpf embryos. The position of the DLE is highlighted in magenta shading. (B) A representative autoradiogram shows probes spanning sqt 3'UTR when incubated with embryo extract bind to several activities, named as sqt RNA Binding Factors (SRBFs). A shift, SRBF1, is detected on the sqt1 probe which encompasses the DLE. The SRBF1 shift is not detected on the other probes. SRBF2 and SRBF4 bind to multiple probes whereas SRBF3 activity is detected on sqt4. (C) Schematic showing regions of sqt 3'UTR bound by different SRBFs.
[Data from Patrick C. Gilligan]

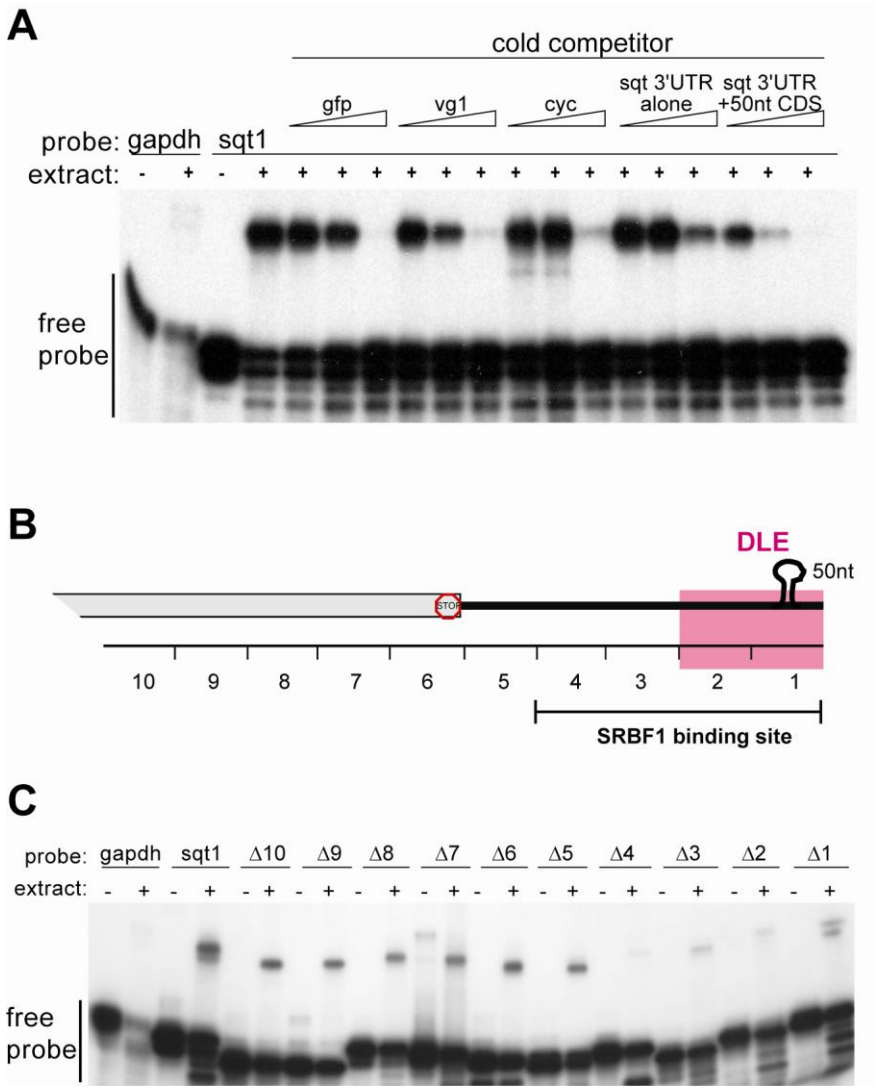


Figure 3.5 – *SRBF1 specifically binds to sqt DLE*
(A) Competition gel shift assay, where ~0.1 ng of radioactively labeled sqt probe is competed against 5-80 ng of unlabeled RNA, shows that SRBF1 binds specifically to sqt RNA. The sqt 3'UTR with 50 nucleotides of coding sequence competes more strongly than the negative control gfp, or control vg1 and cyc RNA for binding to sqt1 probe. Triangles represent increasing amounts of cold competitor RNA. (B) Schematic showing the SRBF1 binding site. Deletions of 10 nucleotides were generated in the 3'UTR and in the coding sequence spanning the sqt1 probe. The sqt DLE is indicated by pink shading. Red octagon indicates the stop codon. (C) RNA gel shifts were performed with the sqt1 deletion series. Δ1-Δ4 reduce/abolish SRBF1 binding to sqt1. The SRBF1 binding site overlaps with the DLE.
[Data from Patrick C. Gilligan]

3.2.3 Purification of sqt RNA binding factor 1 (SRBF1)

In order to purify the factors that bind to the sqt 3'UTR, we fractionated zebrafish embryo extracts by column chromatography. We tested a number of chromatographic columns to find whether the protein of our interest binds to it and designed a purification strategy. The size of a specific binding factor was determined by UV crosslinking assays. SRBF1 was purified by fractionating zebrafish embryo extracts on heparin and hydrophobic interaction columns (Figure 3.6A). Individual fractions were screened for SRBF1 activity by gel mobility-shift and UV cross-linking assays (Figure 3.6B, C). UV cross-linking shows that SRBF1 runs at ~50kDa (Figure 3.6B). A ~50 kDa coomassie staining factor co-fractionated with the SRBF1 activity (Figure 3.6C, D), suggesting that this may be SRBF1. The ~50 kDa band was excised and identified by mass spectrometry to contain the conserved nucleic acid binding protein, Y box-binding protein 1 (Ybx1). Ybx1 has a predicted molecular weight of 36 kDa, but mammalian Ybx1 is reported to run at ~ 48–50 kDa on SDS PAGE gels (Evdokimova et al., 1995).

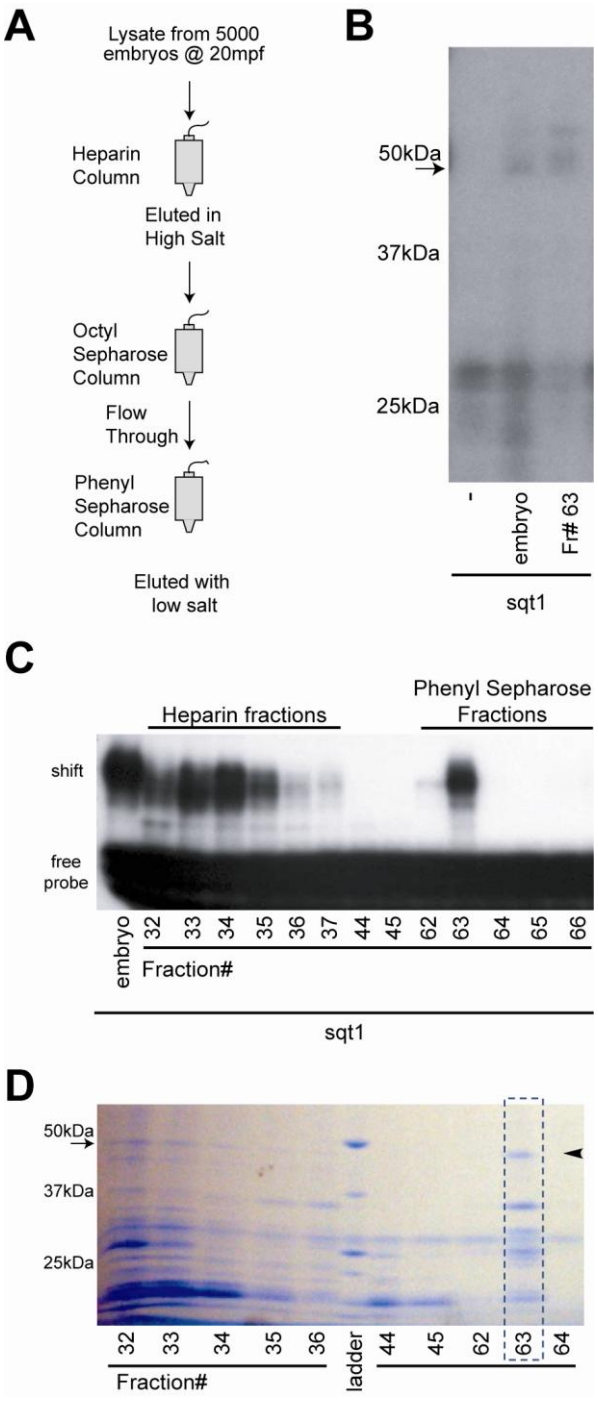

A
Lysate from 5000 embryos @ 20mpf
Heparin Column
Eluted in High Salt
Octyl Sepharose Column
Flow Through
Phenyl Sepharose Column
Eluted with low salt
B
50kDa
37kDa
25kDa
-
embryo
Fr# 63
sqt1
C
Heparin fractions
Phenyl Sepharose Fractions
shift
free probe
embryo
32
33
34
35
36
37
44
45
62
63
64
65
66
Fraction#
sqt1
D
50kDa
37kDa
25kDa
32
33
34
35
36
ladder
44
45
62
63
64
Fraction#

Figure 3.6 – *Purification of SRBF1*

(A) 5000 embryos were collected at 20mpf for chromatographic purification of SRBF1. Embryo extracts were fractioned on multiple columns sequentially until SRBF1 was partially pure. After each fractionation, all fractions were tested for SRBF1 activity and positive fractions were pooled and loaded onto the next column for further purification. (B) RNA crosslinking shows SRBF1 runs at ~50 kDa on a SDS-PAGE gel. Fraction # 63 shows a similar sized activity. (C) A representative autoradiogram showing SRBF1 purification. SRBF1 activity is detected in fractions 32-37 from heparin column and fractions 62-63 from phenyl sepharose column. (D) A Coomassie-blue stained SDS-PAGE gel of the fractions in C show a ~50 kDa band that co-fractionates with SRBF1 (black arrowhead in fraction#63). The 50 kDa band from fraction#63 was excised, sequenced by mass spectrometry, and found to contain Ybx1 peptides.
[Data from Patrick C. Gilligan]

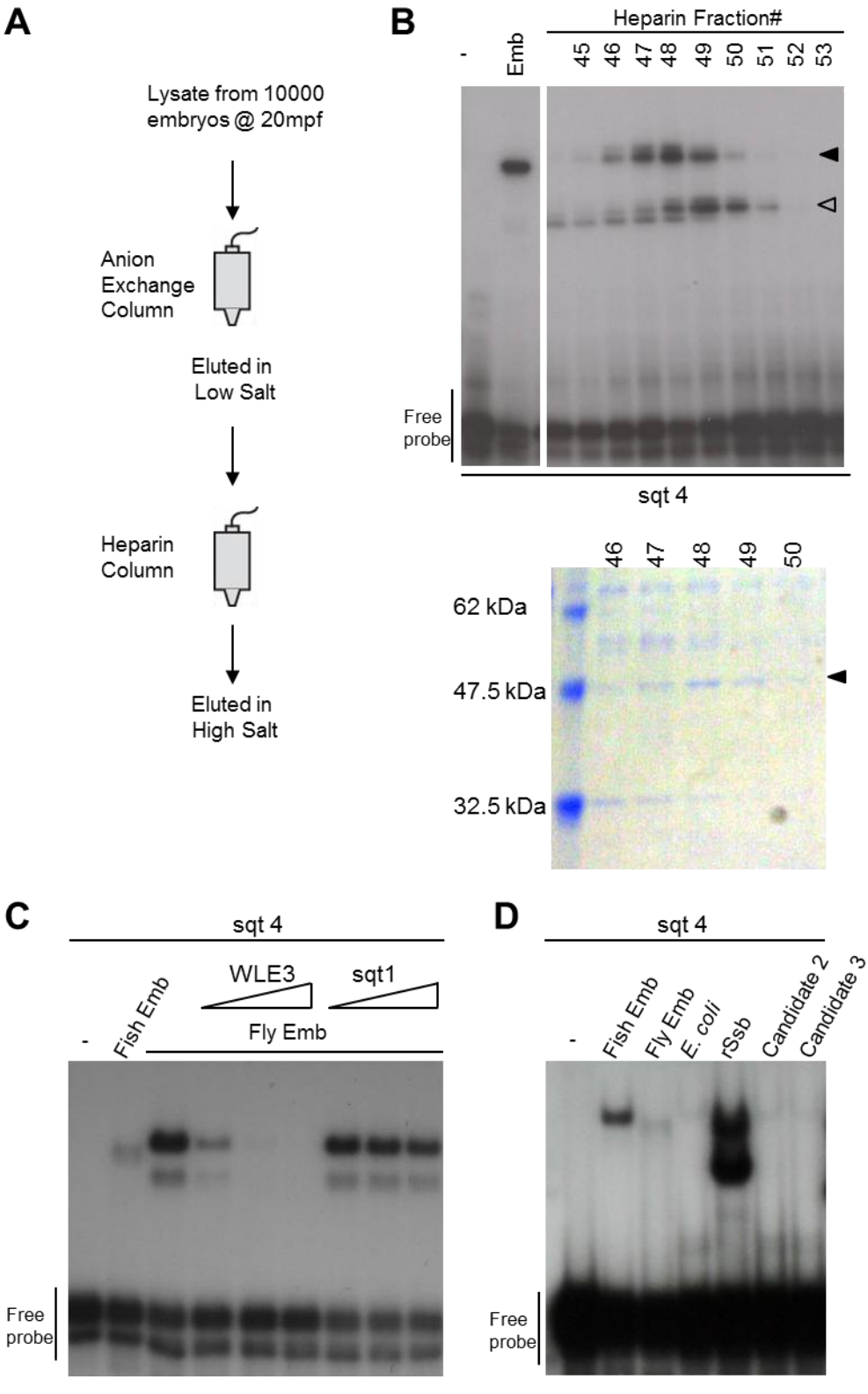
3.2.4 Purification of sqt RNA binding factor 3 (SRBF3)

SRBF3, which bind to sqt4 region of sqt 3'UTR (Figure 3.4B, C), was purified using a similar strategy. 10 mg of zebrafish embryo extracts were fractionated on an anion exchange column. Each fraction was tested for SRBF3 activity by RNA gel shift assays. SRBF3 was eluted in low salt conditions from this column. Positive fractions were pooled, dialyzed and further fractionated using a heparin column and SRBF3 was eluted with high salt buffer (Figure 3.7A, B). Positive fractions were concentrated and analyzed on a 6-20% gradient SDS-PAGE. A 47.5 kDa factor co-fractionating with the SRFB3 activity was observed after coomassie staining (black arrowheads, Figure 3.7B). Mass spectrometry analysis showed that this band contains peptides of the RNA binding protein Sjogren Syndrome Antigen B (Ssb), also known as Autoantigen La.

SRBF3 activity was observed in gel shift assays with fly embryo lysates also. Interestingly a localization element from 3' UTR of *Drosophila* wingless RNA, WLE3 (dos Santos et al., 2008) completes against sqt4 for SRBF3 binding for which sqt1 does not compete (Figure 3.7C). WLE3 confers apical localization in *Drosophila* embryos.

To confirm SRBF3 is Ssb/Autoantigen La, I cloned zebrafish *ssb* coding sequences and expressed recombinant Ssb/La protein in *E.coli* for use in RNA-binding experiments. Recombinant Ssb, but not other candidates as suggested by mass spectrometry analysis, binds to sqt4 probe similar to the embryo extracts. (Figure 3.7D).

As SRBF1 is the DLE-binding factor, I chose to characterize SRBF1 for the remainder of my thesis work.


A
Lysate from 10000 embryos @ 20mpf
Anion Exchange Column
Eluted in Low Salt
Heparin Column
Eluted in High Salt
B
Heparin Fraction#
-
Emb
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48
49
50
51
52
53
Free probe
sqt 4
46
47
48
49
50
62 kDa
47.5 kDa
32.5 kDa
C
sqt 4
WLE3
sqt1
-
Fish Emb
Fly Emb
Free probe
D
sqt 4
-
Fish Emb
Fly Emb
E. coli rSsb
Candidate 2
Candidate 3
Free probe

Figure 3.7 – *Purification of SRBF3*

(A) 10000 embryos were collected at 20 mpf for chromatographic purification of SRBF3. Embryo extracts were fractioned on various columns sequentially, until SRBF3 was partially pure. After each step fractions were tested for SRBF3 activity and positive fractions were pooled and loaded onto the next column for further purification. (B) A representative autoradiogram showing SRBF3 purification. SRBF3 activity is detected in fractions 45-50 from the heparin column (black arrowhead). Lower bands indicated by an open arrowhead might be a degradation product. Coomassie-blue stained SDS-PAGE gel of the fractions 45-50 in the bottom panel shows a ~47.5 kDa band that co-fractionates with SRBF3 (black arrowhead). The 47.5 kDa band from fraction#49 was excised, sequenced by mass spectrometry. (C) SRBF3 activity was also found in fly embryo lysates. The fly wingless localization element 3 (WLE3), but not sqt1 probe, competes with the sqt4 probe, suggesting that WLE and sqt4 bind the same activity. (D) rSsb shows a shift similar to SRBF3 whereas other candidates obtained from mass spectrometry analysis of ~47.5 kDa band from fraction #49 in B do not bind sqt4.
[Data in panel C is from Patrick C. Gilligan]

3.2.5 SRBF1 is the nucleic acid binding protein Y box-binding protein1 (Ybx1)

Ybx1, a nucleic acid binding protein, is a member of a large family of proteins with an evolutionarily conserved cold-shock domain defined by its similarity to the bacterial cold shock proteins CspA and CspB (Eliseeva et al., 2012; Kohno et al., 2003). There are two conserved RNA binding motifs in Ybx1 – RNP1 and RNP2, and a single stranded DNA binding domain (ssDBD). The N-terminus of Ybx1 also contains an actin-binding domain (Ruzanov et al., 1999), which may be conserved in *Drosophila* Ypsilon schachtel (Yps), a dimerization domain (Izumi et al., 2001), and a non-canonical Nuclear Localization Signal (NLS; (Bader and Vogt, 2005)), which are both conserved amongst the vertebrate Ybx1 proteins, but do not appear to be conserved in *Drosophila* Yps (Figure 3.8). Ybx1 is a multifunctional protein with roles in transcriptional regulation, pre-mRNA splicing, mRNA stabilization, transport and translational regulation (Eliseeva et al., 2012; Kohno et al., 2003; Raffetseder et al., 2003; Tanaka et al., 2004; Tanaka et al., 2010). Thus, Ybx1 functions in global as well as specific gene regulation at various levels.

To confirm that Ybx1 is SRBF1, zebrafish *ybx1* cDNA sequences were cloned, recombinant Ybx1 (rYbx1) was expressed in *E. coli*, and tested for sqt DLE-binding activity. Endogenous SRBF1 from zebrafish embryos and 6XHis-tagged rYbx1 bind to sense sqt1 probe, but not to control gapdh, or antisense sqt1 probes (Figure 3.9A). In addition, recombinant Ybx1 (rYbx1) competes with SRBF1 for binding to the sqt1 probe (Figure 3.9B). Thus, bacterially expressed rYbx1 can bind sqt DLE sequences with the same specificity as embryonic SRBF1. The shift formed by rYbx1 has higher mobility on native gels, possibly because it lacks post-translational modifications or binding partners that may be present in zebrafish eggs and embryos.

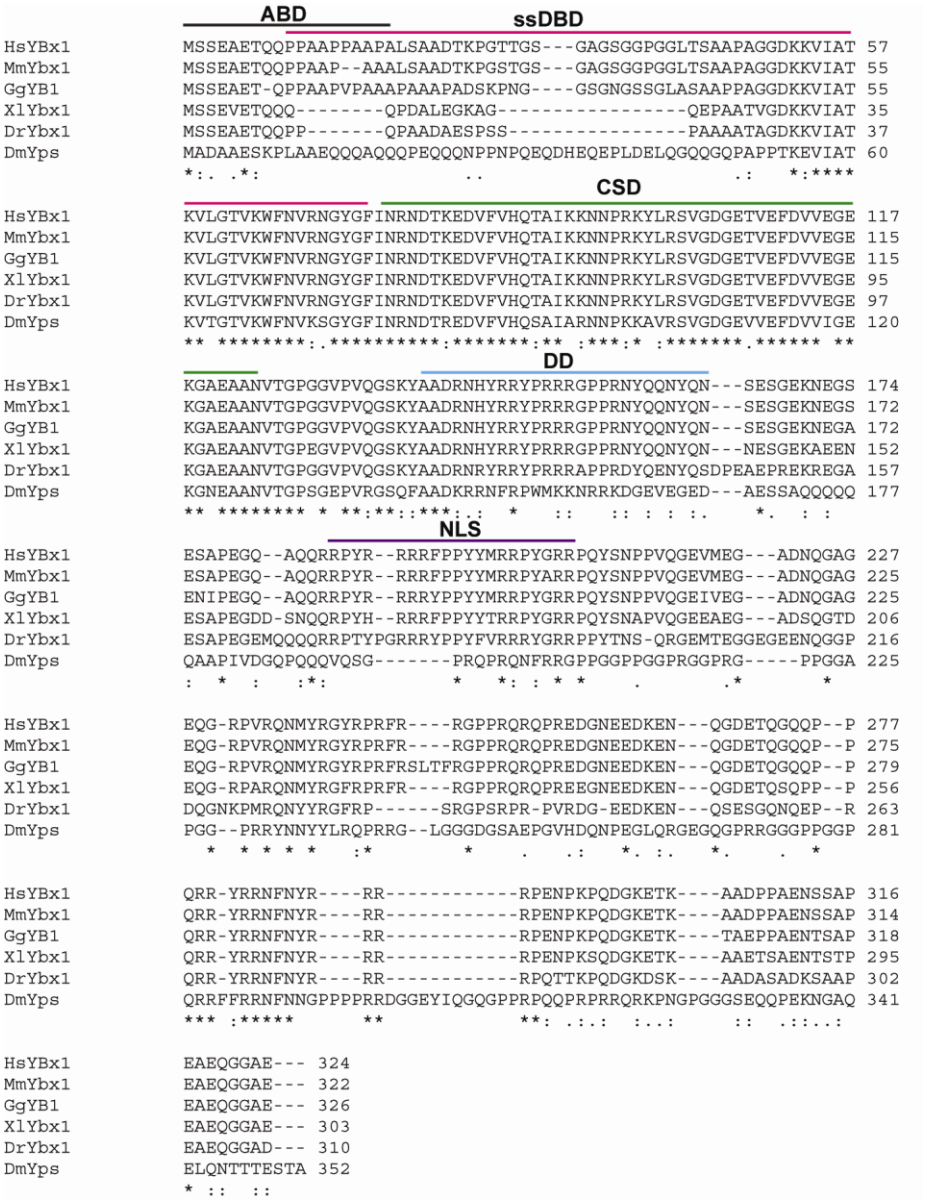


Figure 3.8 – *Alignment of Ybx1 sequences from different species.*

Alignment of Ybx1 sequences. The actin binding domain (ABD), single stranded DNA-binding domain (ssDBD), cold shock domain (CSD), dimerization domain (DD), and nuclear localization sequence (NLS) are indicated. Species names and Genbank Accession numbers are as follows: *Homo sapiens*, AAI06046.1; *Mus musculus*, AAH61634.1; *Gallus gallus*, NM_204414.1; *Danio rerio*, AAI68507.1; *Xenopus laevis*, AAH41191.1; *Drosophila melanogaster*, NM_079309.3.

3.2.6 Ybx1 forms protein-RNA complex *in vivo* with sqt RNA

In order to test if Ybx1 forms protein-RNA complexes *in vivo* with sqt RNA, I performed RNA-immunoprecipitation (RNA-IP) using 20 mpf embryo lysates. Immunoprecipitated samples were subjected to RT-PCR for detecting sqt. RNA-IP with anti-Ybx1 antibodies pulled down sqt RNA but not control gapdh and wnt8a RNA. RNA-IP using IgG antibodies did not show any sqt product (Figure 3.9C). Therefore, Ybx1 specifically binds to sqt RNA in early embryos.

3.2.7 Recombinant Ybx1 binds sqt DLE but nor wnt8a or vg1 3' UTRs

Ybx1 has also been reported to bind RNA in a sequence non-specific manner (Izumi et al., 2001; Kohno et al., 2003). Hence, to determine the specificity of Ybx1 binding to sqt, I performed gel shift assays with probes derived from UTRs of other localized RNAs. Vg1 RNA localizes to the animal pole of stage IV oocytes (Bally-Cuif et al., 1998) and wnt8a RNA is asymmetrically localized at the vegetal cortex of 2 -8 cell stage zebrafish embryos (Lu et al., 2011). The probes were designed in a manner similar to the sqt probes (Figure 3.4A). The probes spanning vg1 and wnt8a 3'UTRs do not bind to rYbx1 (Figure 3.9D).

3.2.8 Ybx1 binding to DLE requires additional elements in sqt coding sequence

For RNA gel shifts, we used overlapping probes spanning the sqt 3' UTR. Two probes – sqt1 and sqt2 harbor the sqt DLE. Ybx1 binds strongly to sqt1 but does not bind as well to sqt2. This suggests that sequence and/or structural elements present in sqt1, but absent in sqt2, are required for efficient binding of Ybx1 to the DLE. The predicted secondary structure of this region of the RNA (sqt 1-2, Figure 3.10A) suggests that some part of coding sequence might be required for the RNA to fold correctly. We tested this by elongating the sqt2 probe with additional 20 bases from the coding sequence and found that this

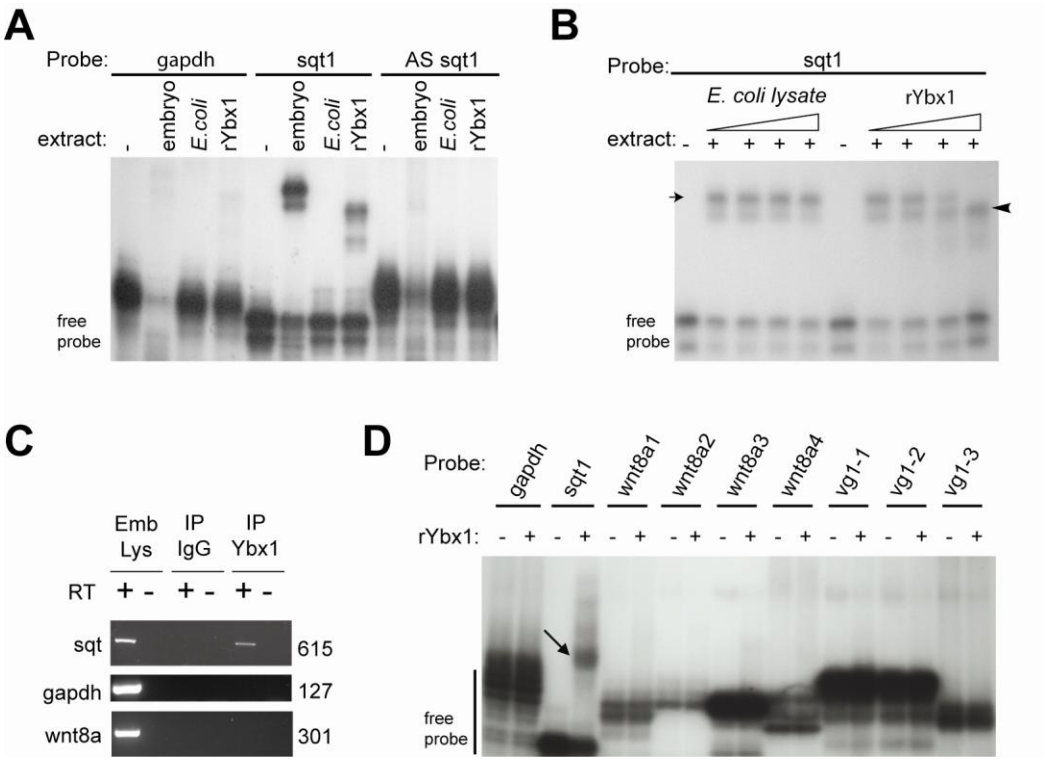


Figure 3.9 – *SRBF1 is the conserved nucleic acid binding protein Ybx1.*

(A) Recombinant Ybx1 (rYbx1) binds to DLE containing probe sqt1 and the shift is similar to the SRBF1 activity from embryo lysates. In contrast sqt1 does not bind to antisense sqt1 or gapdh probes. (B) Gel shift assay shows that rYbx1 (black arrowhead) competes with endogenous Ybx1 (arrow) for binding to the sqt1 probe. Triangles indicate 5-fold increments of *E. coli* lysate or rYbx1. (C) RNA-Immunoprecipitation with anti-Ybx1 antibodies followed by RT-PCR shows that Ybx1 binds to sqt RNA but not gapdh or wnt8a *in vivo*. Control IgG antibodies do not pull down sqt RNA. RT-PCR from whole embryo lysates is the positive control. PCR product sizes are indicated on the right. (D) RNA gel shifts with probes spanning 3'UTR of wnt8a (wnt8a 1-4) and vg1 (vg1 1-3) show that rYbx1 binds to sqt1 (black arrow) but not wnt8a, vg1 or gapdh probes.

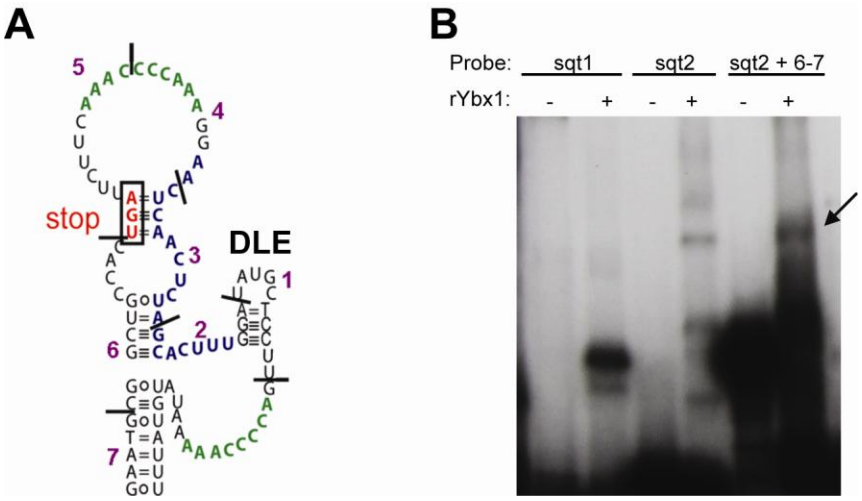


Figure 3.10 – *Ybx1 binding requires elements in sqt coding sequence.*

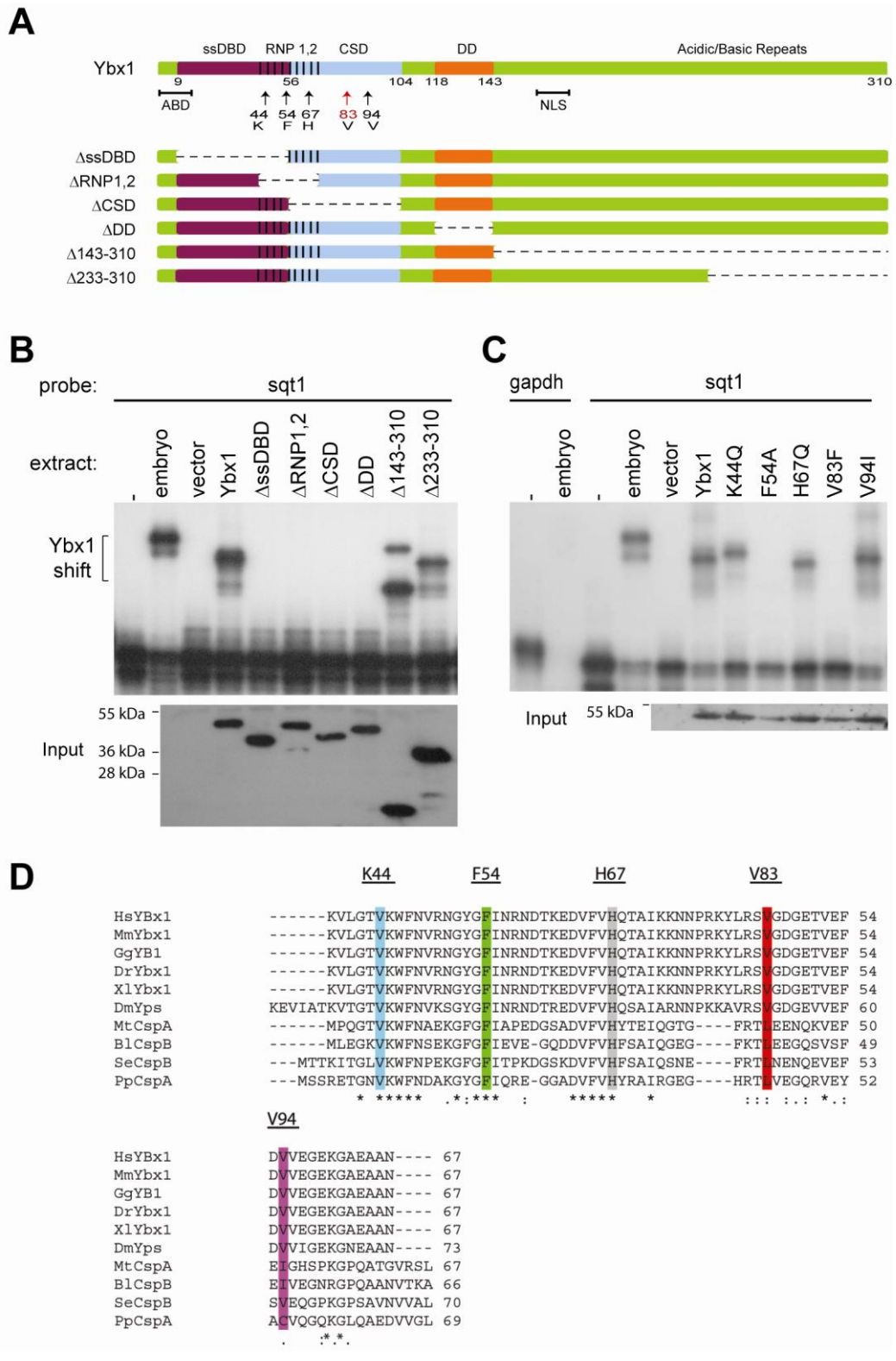
(A) Predicted secondary structure of sqt 3'UTR containing the regions in sqt1 and sqt2 probes. (B) Recombinant Ybx1 binds weakly to the sqt2 probe, but sqt2 with some coding sequences (regions 6 and 7) shows stronger binding.

extension improved its binding to Ybx1 (Figure 3.10B). Thus, efficient binding of Ybx1 to the sqt DLE requires additional elements in the sqt coding sequences.

3.2.9 The N-terminus of Ybx1 is required for binding sqt RNA

The Ybx1 binding site in the sqt DLE contains a conserved single stranded motif AGCAC which is somewhat similar to previously described Ybx1 consensus sequences (Bouvet et al., 1995; Giorgini et al., 2001; Zasedateleva et al., 2002), and a hairpin (Gilligan et al., 2011) . It has been suggested that the acidic/basic rich C-terminal half of Ybx1 is also involved in RNA binding (Izumi et al., 2001). So, we wanted to know which residues of Ybx1 are involved in binding the sqt DLE sequence. We made a series of deletions that removed each of the various domains (Single stranded DNA binding domain, ssDBD; Cold shock domain, CSD; RNP 1,2; dimerization domain, DD) individually, and one that removes the entire C-terminal half of the protein (Figure 3.8 and Figure 3.11A). We find that the C-terminal half of the protein containing the NLS is dispensable for sqt RNA binding (Figure 3.11A, B). By contrast, deletions in ssDBD, RNP1,2 and CSD abolish RNA binding. Mutations in the DD also affect Ybx1 binding to sqt RNA (Figure 3.11A, B).

We next made point mutations affecting conserved amino acid residues in the RNA binding domains of Ybx1 (Figure 3.11A, C, D). K44, F54 and H67 were selected on the basis of predicted NMR structure of bacterial cold shock proteins (Manival et al., 2001; Schroder et al., 1995) and human Yb-1 (Kloks et al., 2002) which suggest that these residues make contact with nucleic acid. V83 and V94 residues were found to be mutated in a Zebrafish ENU induced mutant bank (TILLING bank). The point mutations F54A and V83F abolish binding of rYBx1 to sqt1 probe (Figure 3.11C). H67Q, K44Q and V94I mutations did not affect the binding at the concentrations used and the mutant protein was still able to bind to the DLE-containing probe (Figure 3.11C). These results indicate that Ybx1 binds the sqt DLE via its RNA binding domains in the N-terminus.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RNP 1,2
CSD
DD
Acidic/Basic Repeats
Ybx1
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56
104
118
143
310
ABD
NLS
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54 F
67 H
83 V
94 V
ΔssDBD
ΔRNP1,2
ΔCSD
ΔDD
Δ143-310
Δ233-310
B
probe:
sqt1
extract:
-
embryo
vector
Ybx1
ΔssDBD
ΔRNP1,2
ΔCSD
ΔDD
Δ143-310
Δ233-310
Ybx1 shift
55 kDa
Input
36 kDa
28 kDa
C
gapdh
sqt1
-
embryo
-
embryo
vector
Ybx1
K44Q
F54A
H67Q
V83F
V94I
Input
55 kDa
D
K44
F54
H67
V83
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MmYbx1 ------KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF 54
GgYB1 ------KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF 54
DrYbx1 ------KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF 54
XlYbx1 ------KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF 54
DmYps KEVIATKVTGTVKWFNVKSGYGFINRNDTREDVFVHQSAIARNNPKKAVRSVGDGEVVEF 60
MtCspA ------MPQGTVKWFNAEKGFGFIAPEDGSADVFVHYTEIQGTG----FRTLEENQKVEF 50
BlCspB ------MLEGKVKWFNSEKGFGFIEVE-GQDDVFVHFSAIQGEG----FKTLEEGQSVSF 49
SeCspB ---MTTKITGLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSNE----FRTLNENQEVEF 53
PpCspA ---MSSRETGNVKWFNDAKGYGFIQRE-GGADVFVHYRAIRGEG----HRTLVEGQRVEY 52
V94
HsYBx1 DVVEGEKGAEAAN---- 67
MmYbx1 DVVEGEKGAEAAN---- 67
GgYB1 DVVEGEKGAEAAN---- 67
DrYbx1 DVVEGEKGAEAAN---- 67
XlYbx1 DVVEGEKGAEAAN---- 67
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BlCspB EIVEGNRGPQAANVTKA 66
SeCspB SVEQGPKGPSAVNVVAL 70
PpCspA ACVQGQKGLQAEDVVGL 69

Figure 3.11 – *Ybx1 binds sqt DLE via its RNA binding domain*

(A) Schematics showing Ybx1 structure and deletion constructs (drawn to scale). The positions of various domains are marked and amino acid substitutions are indicated by arrows. The actin binding domain (ABD), single stranded DNA-binding domain (ssDBD), RNA binding domains (RNP1,2), cold shock domain (CSD), dimerization domain (DD) and Nuclear Localization Signal (NLS) are shown. Hashed lines indicate deletions and numbers indicate amino acid residue. (B) The N-Terminus of Ybx1 containing the ssDBD, CSD and RNP1,2 is required for binding to sqt1. The dimerization domain also plays a role in sqt1 binding. In contrast, the C-Terminus (144-310) is dispensable for sqt1 binding. Expression of different Ybx1 deletion proteins is shown by a western blot with anti-His antibody. (C) Point mutations in Ybx1 identify key amino acid residues that are essential for sqt RNA binding. F54A abolishes binding, whereas K44Q and H67Q do not affect binding at the protein concentrations used. V83F, a mutation identified in zebrafish mutagenesis screen, abolishes sqt1 binding, whereas V94I mutation does not affect sqt1 binding. Western blot with α-His tag antibodies shows expression of mutant Ybx1 proteins. (D) Alignment of cold shock proteins from bacterial species with eukaryotic CSD-containing proteins. The K44, F54, and H67 highlighted residues were identified by NMR to contact RNA. The V83 and V94 residues that were mutated by ENU (identified by TILLING) are also highlighted. Species name and GenBank Accession numbers for bacterial proteins are *Pseudomonas putida*, ADR61621.1; *Mycobacterium tuberculosis*, CCE39069.1; *Salmonella enterica*, CAA72682.1; *Bacillus licheniformis*, AAU39879.1

3.3 Maternal Ybx1 is essential for early development

3.3.1 ybx1 RNA and protein is not spatially restricted

I performed RT-PCR and whole mount *in situ* hybridization to determine the expression profile of ybx1 RNA. ybx1 RNA is expressed maternally and the levels increase after zygotic transcription begins. Expression is not spatially restricted and is detected at all stages of embryogenesis (Figure 3.12A, B). Western blots with anti-Ybx1 antibodies also show maternal and zygotic expression of Ybx1 protein (Figure 3.12C).

3.3.2 *ybx1* mutant alleles

In order to study the role of Ybx1 in embryonic patterning and specifically in sqt RNA localization we screened for mutations in the *ybx1* locus in the ENU-induced mutant bank by TILLING (McCallum et al., 2000). In the TILLING screen, two *ybx1* mutations, *ybx1*^{V83F} (henceforth referred to as *ybx1*^{sa42}) and *ybx1*^{V94I} were identified (Figure 3.13A). RNA gel shift assays with recombinant mutant proteins show that Ybx1^{V83F} lack detectable binding to the sqt-DLE, whereas sqt DLE-binding by Ybx1^{V94I} is similar to wild-type Ybx1 (Figure 3.11C and Figure 3.13B). Gel shift experiments with increasing concentration of recombinant protein show that at very high concentration (~16 fold of wild type rYBx1) rYbx1^{V83F} binds to sqt1 probe (Figure 3.13C). Taken together, the V83F missense mutation significantly reduces the sqt RNA binding activity of Ybx1 protein. Lysates from *ybx1*^{sa42} homozygous embryos lack detectable sqt RNA binding (Figure 3.13D). Western blot analysis on wild-type and mutant embryos lysates with anti-Ybx1 antibody show that mutant embryos have reduced level of Ybx1 protein (Figure 3.13E).

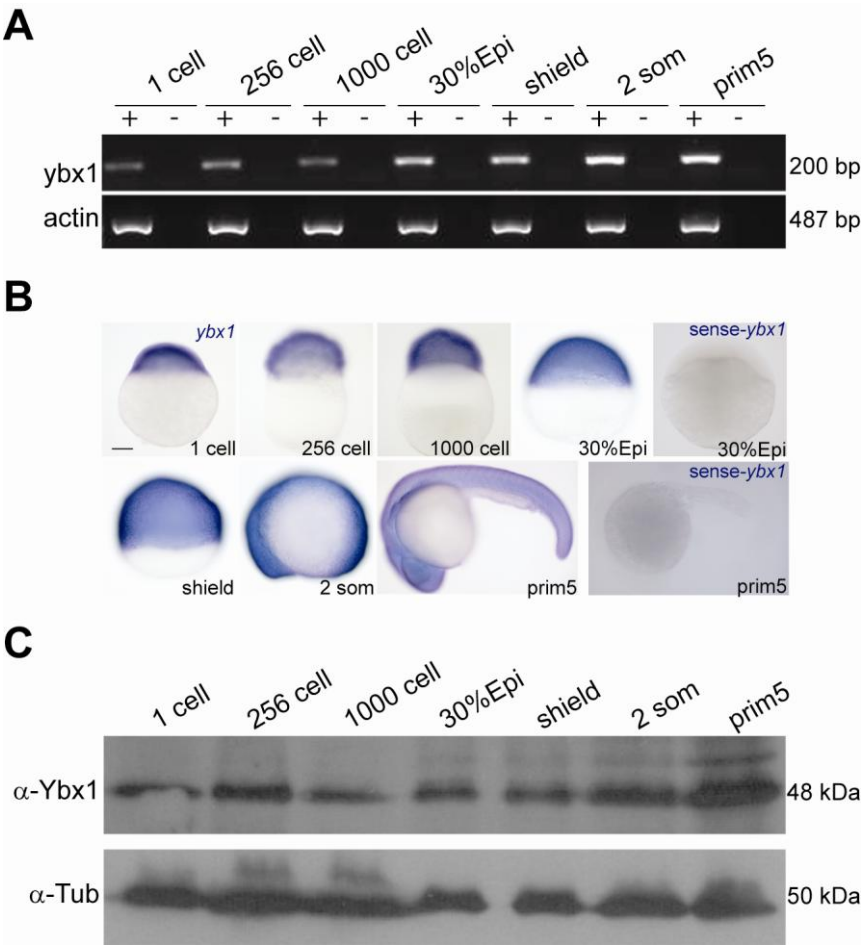

Figure 3.12 – *Expression of ybx1RNA and Ybx1 protein in wild-type embryos.*

(A) Semi-quantitative RT-PCR shows that ybx1 RNA is expressed maternally and expression level increases after zygotic transcription is turned on at mid-blastula transition. Expression of actin serves as a normalization control. (B) RNA i*n situ* hybridization show expression of ybx1 is not spatially restricted. (C) Western blot with anti-Ybx1 antibody shows maternal and zygotic expression of Ybx1.Tubulin is detected as control. Scale bar, 100 µm.

I also generated deletions in the *ybx1* locus by using a pair of zinc-finger nucleases (ZFN) targeting the exon 5 of *ybx1* (Amacher, 2008; Doyon et al., 2008; Meng et al., 2008). I screened 55 injected fishes and identified 3 alleles (Table 3.1). All the three mutations (*ybx1*^{sg5}, *ybx1*^{sg7}, *ybx1*^{sg8}) will lead to a frameshift, followed by premature stop codon. In this study, we use the ZFN allele, *ybx1*^{sg8} which has a 5 bp deletion in exon 5 leading to frame-shift after amino acid residue 197 and premature stop codon at residue 205, resulting in a truncated Ybx1 protein lacking the C-terminus (Ybx1^{sg8}; Figure 3.13A). In contrast to Ybx1^{V83F}, recombinant Ybx1^{sg8} protein (rYbx1$^{\Delta 197-310}$) binds to the sqt DLE and this is consistent with the presence of the CSD in the truncated Ybx1^{sg8} peptide (Figure 3.13B). Thus, *ybx1*^{sa42} affects the RNA-binding CSD of Ybx1, whereas *ybx1*^{sg8} is likely to encode a truncated Ybx1 peptide.

Table 3.1: *Mutations identified in ybx1 locus by zinc finger nuclease injection.*

Deletions are indicated by blue shading and insertion is marked by grey shading.

Mutant	Nature of Mutation	*ybx1* Sequence
ybx1^{sg5}	4 bp deletion	CGCCCCCCCTACACCAACTCACAAAG AGGAGAGATGACAG
ybx1^{sg7}	7 bp insertion	CGCCCCCCCTACACCAACTCCCAACT CACAAAGAGGAGAGATGACAG
ybx1^{s88}	5 bp deletion	CGCCCCCCCTACACCAACTCACAAAG AGGAGAGATGACAG

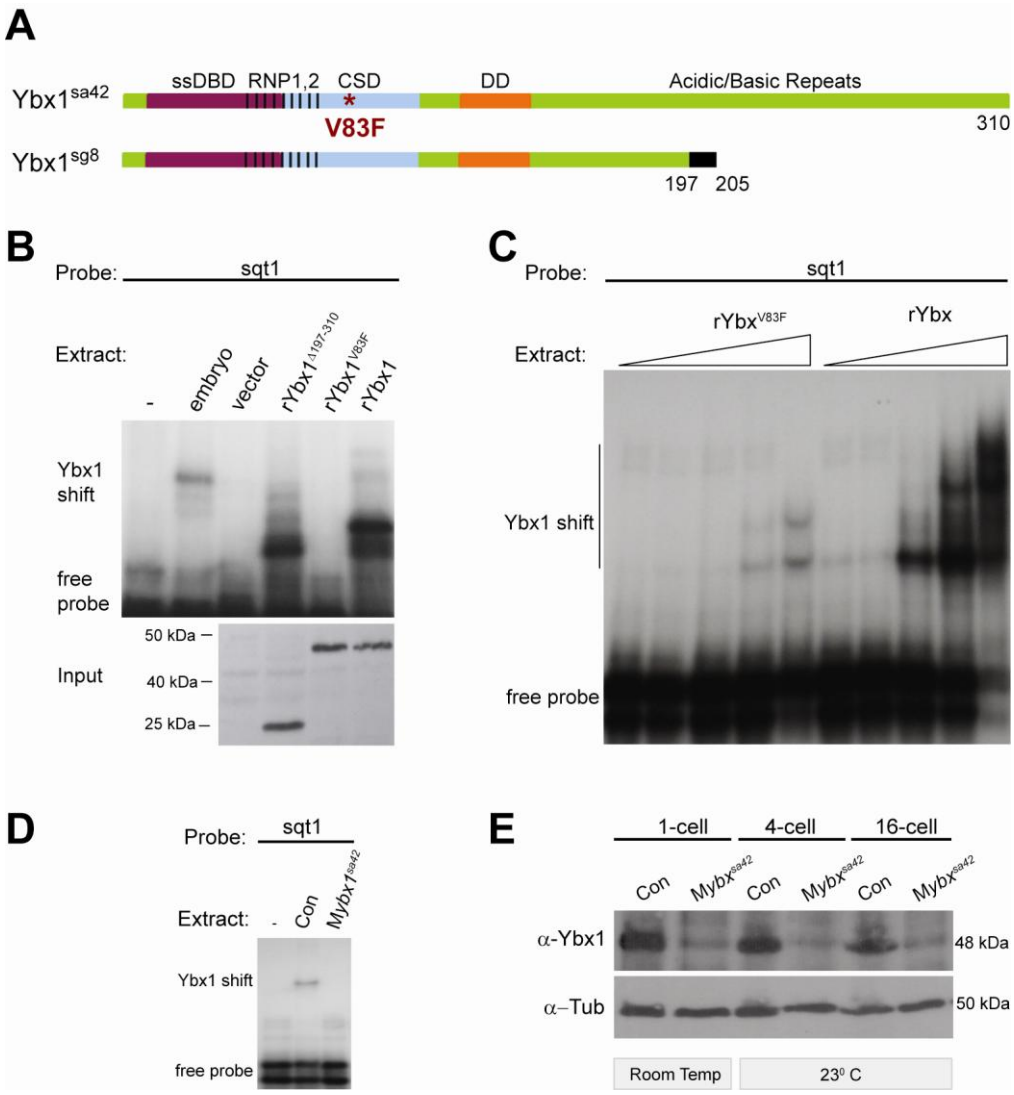

Figure 3.13 – *Ybx1genetics mutants*

(A) Schematic showing the nature of mutations in two genetics *ybx1* mutants – *ybx1*^{sa42}is a missense mutation (V83F) in cold shock domain and *ybx1*^{sg8} is a deletion (Δ197-310) in the C-Terminus of Ybx1. Black block in Ybx1^{sg8} indicated frameshift after residue 197 and premature stop after residue 205. (B) rYbx1^{V83F} lacks detectable binding to sqt1 probe, while rYbx1^{sg8} (rYbx1$^{\Delta 197\text{-}310}$) show binding. Western blot with anti-His antibody shows expression of recombinant Ybx1 proteins. (C) At higher concentration rYbx1^{V83F} shows binding to sqt1. Triangles indicate 5-fold increment in concentrations. (D) M*ybx1*^{sa42} embryos lysates lack detectable binding to sqt1 probe as compared to lysates from wild-type embryos. (E) Western blot with anti-Ybx1 antibody shows M*ybx1*^{sa42} embryos have reduced levels of Ybx1 protein. Tubulin expression is used as loading control.

3.3.3 M*ybx1* mutant embryos fail to initiate gastrulation movements

Homozygous *ybx1*^{sa42} and *ybx1*^{sg8} mutant embryos grow to adulthood. They are viable and fertile at ambient temperature of 28.5^{o}C. So, I obtained maternal mutant embryos by crossing homozygous *ybx1*^{sa42} or *ybx1*^{sg8} females with wild-type males (M*ybx1*, Figure 3.14A). Paternal mutants (P*ybx1*) obtained by crossing homozygous *ybx1*^{sa42} or *ybx1*^{sg8} males with wild-type females were used as controls throughout all the experiments. M*ybx1*^{sa42} mutant embryos develop normally at 28.5^{o}C (Figure 3.14B) and are indistinguishable from wild type or P*ybx1* control embryos. However, at a lower temperature of 23^{o}C, M*ybx1*^{sa42} mutant embryos fail to initiate gastrulation movements. They arrest at the onset of epiboly and fail to survive (Figure 3.14B). Early cell divisions are normal at 23^{o}C, but by early blastula stages, marginal cells in M*ybx1*^{sa42} embryos lose their membranes and a large syncytial layer forms over the yolk cell (Black arrowheads in Figure 3.14B). Embryos from homozygous *ybx1*^{sg8} females (M*ybx1*^{sg8}) divide normally till the 16-cell stage, but subsequent cleavages are aberrant. These embryos fail to develop normally and arrest by blastula stages (Figure 3.14B). Thus, maternal Ybx1 is essential for early embryonic development.

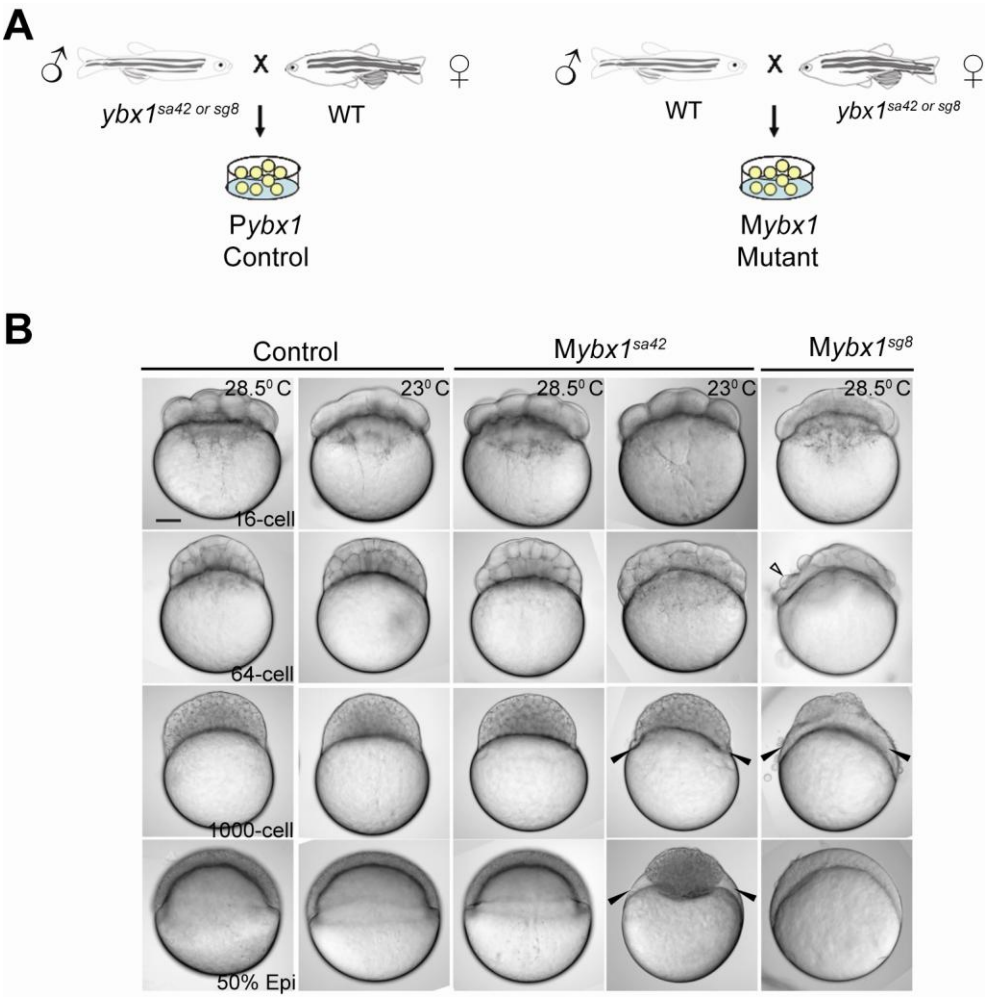

Figure 3.14 – *Maternal Ybx1 is essential for embryogenesis.*

(A) Schematics showing crossing scheme to obtain Paternal (P*ybx1*) and Maternal (M*ybx1)* mutant embryos. P*ybx1* embryos are used as controls against the maternal *ybx1* mutant embryos. (B) DIC images showing control and mutant embryos at 16-cell, 64-cell, 1000-cell and 50% Epiboly stages. M*ybx1*^{sa42} mutant embryos show temperature sensitive gastrulation failure. At 28.5^{o} C, the ambient temperature for zebrafish culture, M*ybx1*^{sa42} mutant embryos are viable. In contrast, at a restrictive temperature of 23^{o} C they fail to initiate gastrulation movements, form an enlarged yolk syncytial layer (black arrowhead) and eventually die. M*ybx1*^{sg8} mutant embryos show normal development until 16-cell stage after which divisions are aberrant and syncytia is formed (open and black arrowheads).

3.3.4 Maternally expressed *ybx1* transgene rescues gastrulation defects in mutants

To rescue the M*ybx1* mutant phenotypes, we injected capped ybx1 mRNA into 1-cell stage mutant embryos. However, RNA injections into embryos failed to rescue M*ybx1* mutant phenotypes (N=82). Hence, we made a rescue transgene in the mutant background using Ac-Ds transposon system (Emelyanov et al., 2006). We generated the transposon plasmid harboring genomic y*bx1* sequences fused with the viral 2a peptide and GFP sequences (Figure 3.15A) and co-injected with Ac transposase RNA into homozygous *ybx1*^{sa42} embryos to generate stable *ybx1-2a-gfp* transgenic lines. Transgene expression was marked by GFP fluorescence in embryos. Zygotic expression of Ybx1-2a-GFP from a paternal *Tg*(*ybx1-2a-gfp)* transgene (P*Tg*) failed to rescue gastrulation arrest in M*ybx1* mutant embryos (Figure 3.15B, C). However, maternal expression of Ybx1-2a-GFP (M*Tg*) from 2 independent transgenic insertions rescued M*ybx1*^{sa42} mutant embryos (Figure 3.15B, C). M*Tg* expression from line#4 and line#6 allowed mutant embryos to initiate and complete gastrulation, and survive (n>200 embryos for each line, Figure 3.15D) till prim5 stage. A small number of P*Tg* expressing mutant embryos initiated gastrulation but failed to survive till prim 5 stage (n=345, Figure 3.15D). These results substantiate that maternal activity of Ybx1 is required for gastrulation.

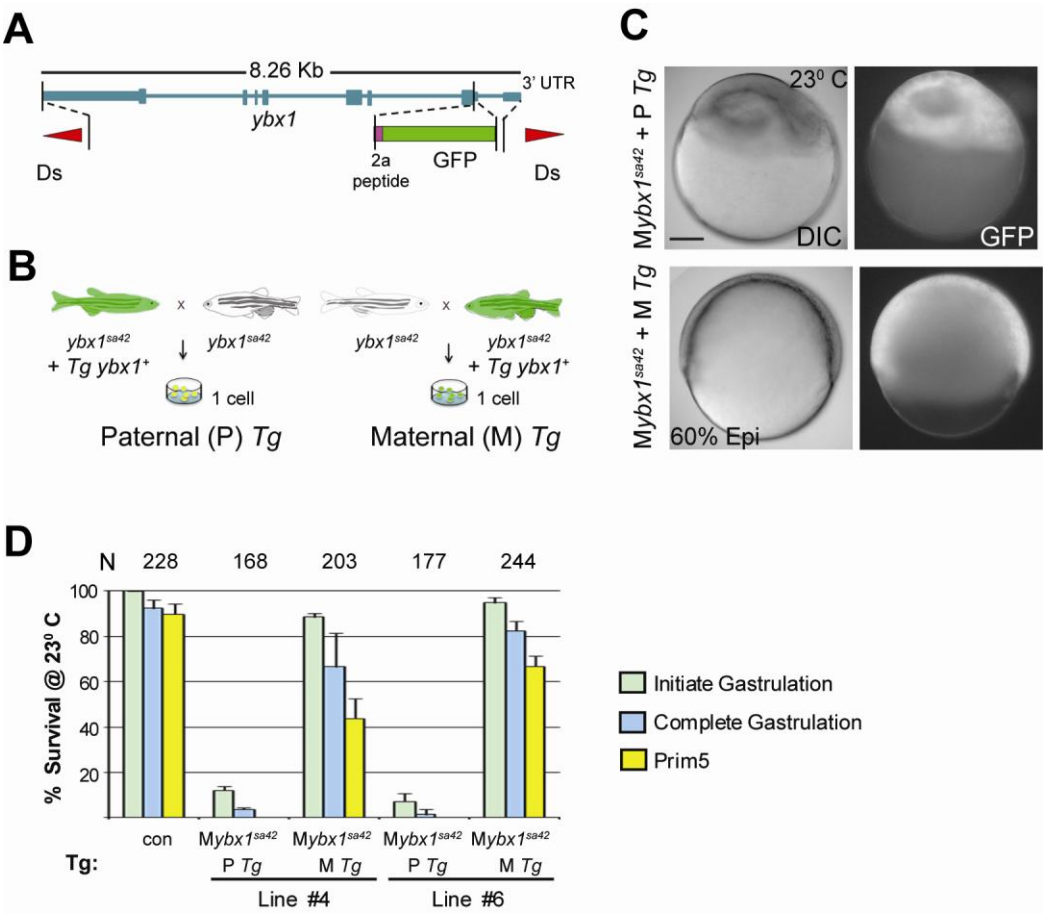

Figure 3.15 – *Maternal ybx1 transgene rescues gastrulation arrest in Mybx1 mutant embryos.*

(A) Schematic representation of *ybx1* genomic locus used for rescue transgene. GFP coding sequence is fused to *ybx1* with a 2a peptide in between the two. Red triangles represent terminal repeats of Ds transposon. (B) Schematics showing crossing scheme to obtain M*ybx1*^{sa42} embryos with either paternal (P*Tg*) or maternal (M*Tg*) *ybx1* transgene. (C) M*ybx1*^{sa42}mutant embryos with maternal expression of wild-type Ybx1 from M*Tg* undergo gastrulation whereas mutant embryos with zygotic expression from P*Tg* fail to gastrulate. (D) Histograms showing initiation and completion of gastrulation and survival till prim5 stage of M*ybx1*^{sa42} mutant embryos with two independent transgenic lines (M *Tg* #4 and M *Tg* #6) at the restrictive temperature of 23^{o} C. A small number of mutant embryos with zygotic expression of Ybx1 from P*Tg* from both lines did initiate gastrulation, but did not survive to prim5. Number of embryos scored is on top of the histograms. Error bar indicate standard deviation from 3 independent experiments.

3.4 sqt RNA localization is disrupted in M*ybx1* embryos

3.4.1 sqt RNA fails to localize to future dorsal in M*ybx1* embryos

Ybx1 was identified as a sqt-DLE binding factor so I performed RNA *in-situ* hybridization to examine spatial distribution of sqt RNA in mutant embryos. At 28.5^{o}C, sqt RNA localization is delayed at the 1-cell stage in M*ybx1*^{sa42} mutant embryos. Nevertheless, by the 4-cell stage, sqt RNA is asymmetrically localized in the blastoderm similar to wild-type embryos (Gore et al., 2005; Gore and Sampath, 2002) and P*ybx1* control embryos (Figure 3.16). However, at the restrictive temperature of 23^{o}C, sqt RNA localization in M*ybx1*^{sa42} mutant embryos is aberrant at 1-cell and 4-cell stages. The RNA does not translocate to blastoderm and remains as aggregates in yolk and eventually fails to localize to the future dorsal cells (Figure 3.16). Localization of sqt RNA is also disrupted in M*ybx1*^{sg8} mutant embryos and sqt RNA remains in the yolk (Figure 3.16).

3.4.2 Maternal *ybx1* transgene rescues sqt RNA localization in mutants

Localization of sqt RNA is restored in M*ybx1*^{sa42} mutant embryos by maternal expression of *ybx1-2a-gfp* transgene (M*Tg*), but not by zygotic expression from a paternally inherited *ybx1* transgene (P*Tg*) (Figure 3.16). Thus, consistent with Ybx1 binding to the sqt DLE, maternal Ybx1 is required for localization of sqt RNA.

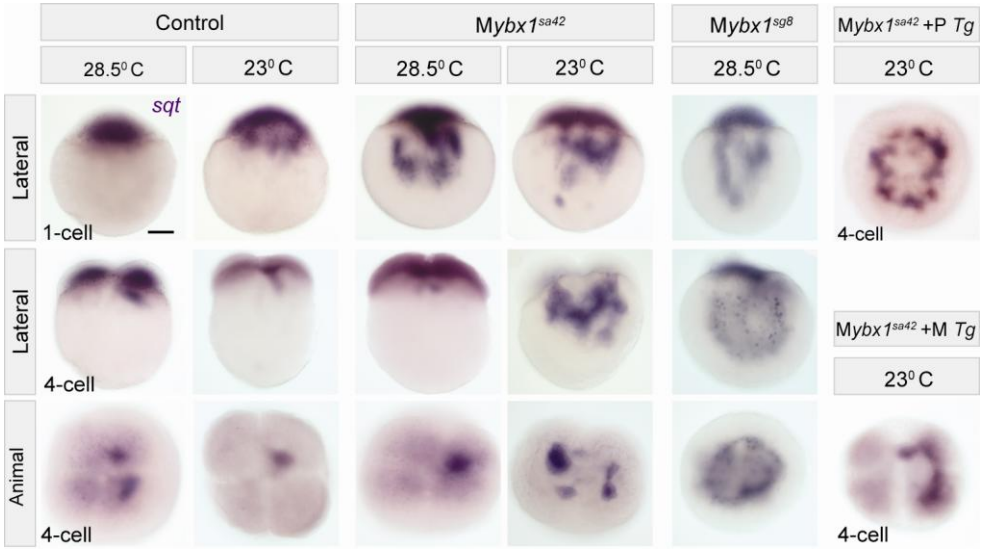

Figure 3.16 – *sqt RNA localization is disrupted in Mybx1 mutant embryos.*

In control embryos, sqt RNA is transported to blastoderm by 1-cell stage and is asymmetrically localized at 4-cell stage at both 28.5° C and 23° C. sqt RNA movement is delayed in 1-cell stage M*ybx1*^{sa42}mutant embryos at 28.5° C but by 4 cell stage the RNA gets asymmetrically localized. At 23° C, sqt RNA forms aggregate in yolk and fails to localize in M*ybx1*^{sa42}mutant embryos. Localization of sqt RNA is also affected in M*ybx1*^{sg8} mutant embryos. Localization of sqt RNA in mutant embryos is restored by maternal expression (M*Tg*) of *ybx1* transgene but not zygotic expression (P*Tg*). Scale bar, 100 μm.

3.4.3 Localization of other transcripts is not affected in M*ybx1* embryos

In order to verify that the disruption of transport in M*ybx1* mutant embryos is specific to sqt, I analyzed the localization of other maternal RNAs. I selected a few RNAs with different localization patterns (Howley and Ho, 2000) during oogenesis, like ubiquitous (snail1a), animal (cyclinB1, eomesodermin), vegetal (wnt8a, grip2) and cortical (vasa). Localization of snail1a, cyclinB1, eomesodermin, grip2, and vasa RNA in M*ybx1* mutant embryos is unchanged at 28.5 or 23^{o}C (Figure 3.17). Expression pattern of maternal wnt8a was also unaffected in M*ybx1*^{sa42} mutant embryos at 23^{o}C (Figure 3.17). In M*ybx1*^{sg8} mutant embryos, vegetal asymmetry and animal pole expression of wnt8a is similar to controls, although I sometimes detected some residual wnt8a in the yolk (open arrowhead in Figure 3.17) in a proportion of embryos (~25%). Taken together, Ybx1 does not affect all RNA transport processes in early embryos. Amongst the various maternal RNAs that I examined, only sqt RNA localization is severely disrupted in M*ybx1* mutant embryos.

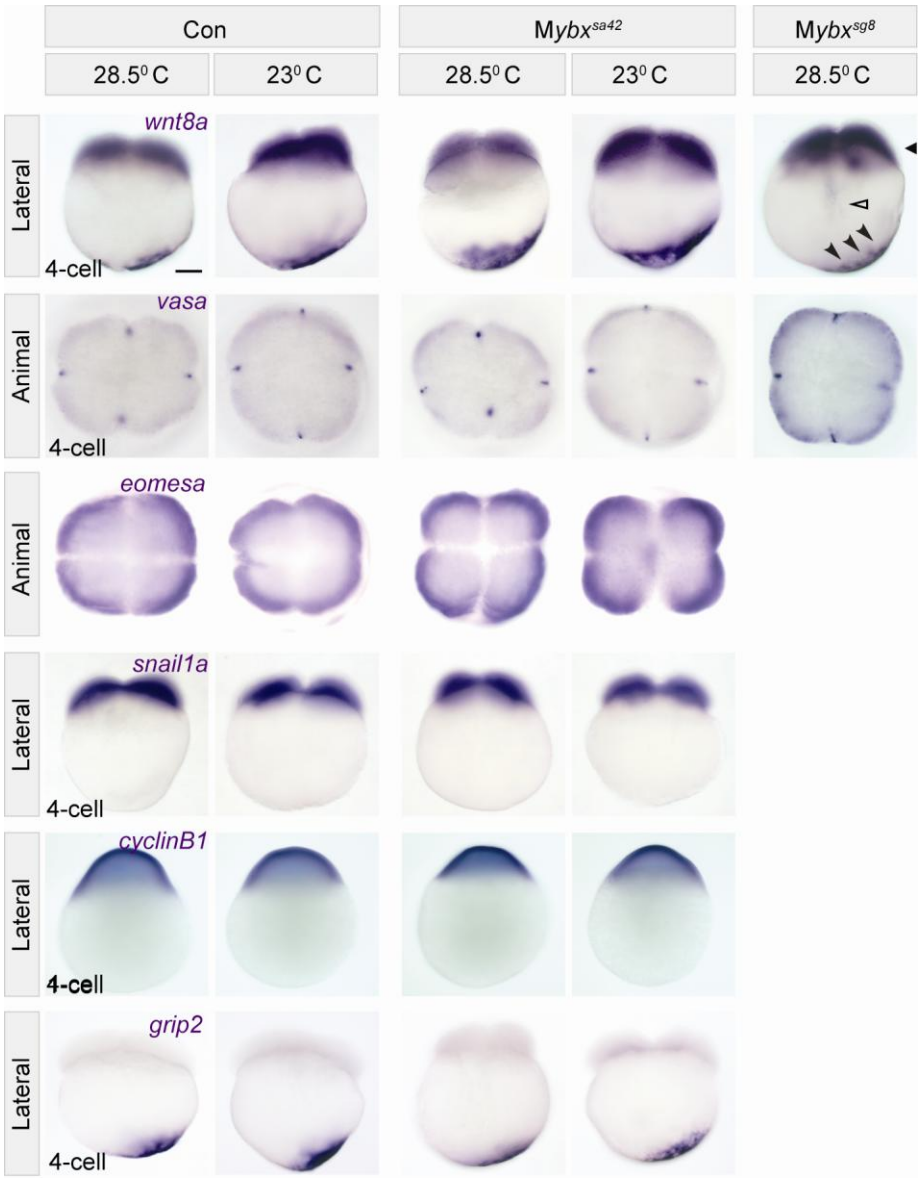

Figure 3.17 – *Localization of other RNAs is not affected in Mybx1 mutant embryos.*

Localization of various maternally expressed RNAs at 1-cell and 4-cell stages is appropriate in M*ybx1* mutant embryos. Vegetal RNAs (wnt8a, grip2), cortical RNAs (vasa, eomesa) and axial streamers (snail1a and cyclinB1) localize correctly in mutant embryos at both 28.5° C and 23° C. In a small proportion of M*ybx1*^{sg8} embryos wnt8a is detected in yolk (open arrowhead) apart from the correct localization pattern in vegetal and animal pole (black arrowheads).

3.5 sqt RNA processing and Sqt translation is precocious in M*ybx1* embryos

In order to determine how mis-localization of sqt may lead to gastrulation defects in M*ybx1* mutant embryos, I performed experiments to analyze the processing of sqt pre-mRNA.

3.5.1 sqt RNA levels are marginally reduced in M*ybx1* embryos

Ybx1 is known to function as a transcriptional (Didier et al., 1988; Dorn et al., 1987), post-transcriptional (Stickeler et al., 2001) and translational regulator (Minich et al., 1993; Ranjan et al., 1993). To determine whether these processes were affected in M*ybx1* mutant embryos, I first examined sqt RNA expression by quantitative real-time PCR (qPCR). QPCR shows that sqt RNA levels are marginally reduced in M*ybx1* mutant embryos in comparison to control embryos (Figure 3.18B).

3.5.2 Polyadenylation and splicing events are accelerated in M*ybx1* embryos

In wild type embryos, sqt is maternally deposited in an unprocessed form i.e. unspliced and non-polyadenylated (Aanes et al., 2011; Lim et al., 2012). The RNA gets completely processed only by the 16 cell stage in wild type embryos. To detect the event of polyadenylation, I performed PCR using oligo-dT primed cDNA samples collected at 1-cell, 4-cell and 16-cell stages. M*ybx1* mutant embryos showed sqt products from the oligo-dT primed cDNA at as early as 1-cell stage whereas the control embryos begin to show poly-A sqt only at 16-cell stage (Figure 3.18C). This indicates precocious polyadenylation of sqt RNA in M*ybx1* mutant embryos. To detect the event of splicing, I performed RT-PCRs with primers that can detect sqt intron 1 and intron 2 (Figure 3.18A and Table 2.1). PCR products from sqt exons (sqt (A-B) and sqt (E-F) in Figure 3.18C) are detected as controls. In comparison to control embryos, PCR products for both introns (E-G and C-D in Figure 3.18C) are reduced/undetectable in M*ybx1* mutant

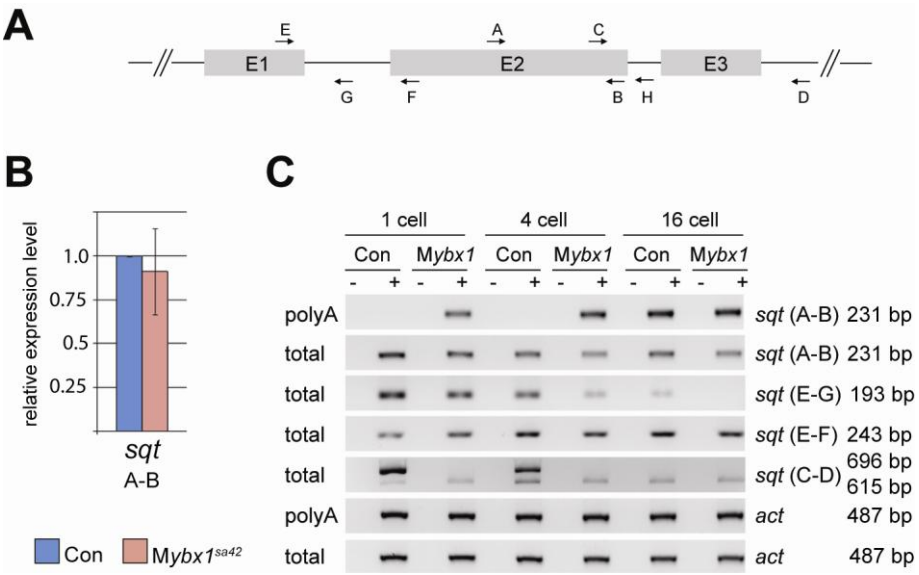

Figure 3.18 – *sqt RNA is processed prematurely in Mybx1 mutant embryos.*

(A) Schematic (not to scale) showing the sqt locus. Exons 1-3 are indicated as E1, E2 and E3. Positions of several primers used are indicated. (B) Quantitative real time PCR show that sqt RNA level is marginally reduced in M*ybx1* mutant embryos. Error bars show standard deviation from 3 biological replicates. (C) Semi quantitative RT-PCR with oligo dT(polyA) and random hexamer (total) primers show that in mutant embryos sqt is polyA tailed at 1-cell stage in contrast to control embryos where polyA tailed sqt is detected only by 16-cell stage. PCR with primers to detect intron 1 (E-G) and intron 2 (C-D) shows that splicing is accelerated in mutant embryos. Actin PCR product is detected as control. Sizes are indicated on right.

embryos at each time point. In conclusion processing of sqt pre-mRNA is precocious in M*ybx1* mutants.

3.5.3 Sqt protein translation is premature in M*ybx1* embryos

As sqt RNA is prematurely processed in M*ybx1* mutant embryos, I then looked into the dynamics of Sqt translation. Due to unavailability of any antibody against Sqt, I used a GFP reporter fused to Sqt. RNA encoding Sqt-GFP fusion protein was injected into 1-cell M*ybx1* mutant embryos, and GFP expression was examined during early blastula stages - 16-cell, 64-cell, 256-cell stages (Figure 3.19A). In M*ybx1* mutants Sqt-GFP expression is detected as early as 16-cell stage, whereas in control embryos, expression of Sqt-GFP is only detected at late blastula stages (Figure 3.19A). Quantification of western blots by density blot analysis show that levels of Sqt-GFP protein is higher in M*ybx1* mutant embryos as compared to control embryos (Figure 3.19B). M*ybx1* mutant embryos when injected with control gfp or wnt8a-gfp RNA (Figure 3.19C, D) do not show deregulated translation. These results suggest that translation of other proteins is not affected in the mutants. Thus, Sqt protein translation is premature in M*ybx1* mutant embryos.

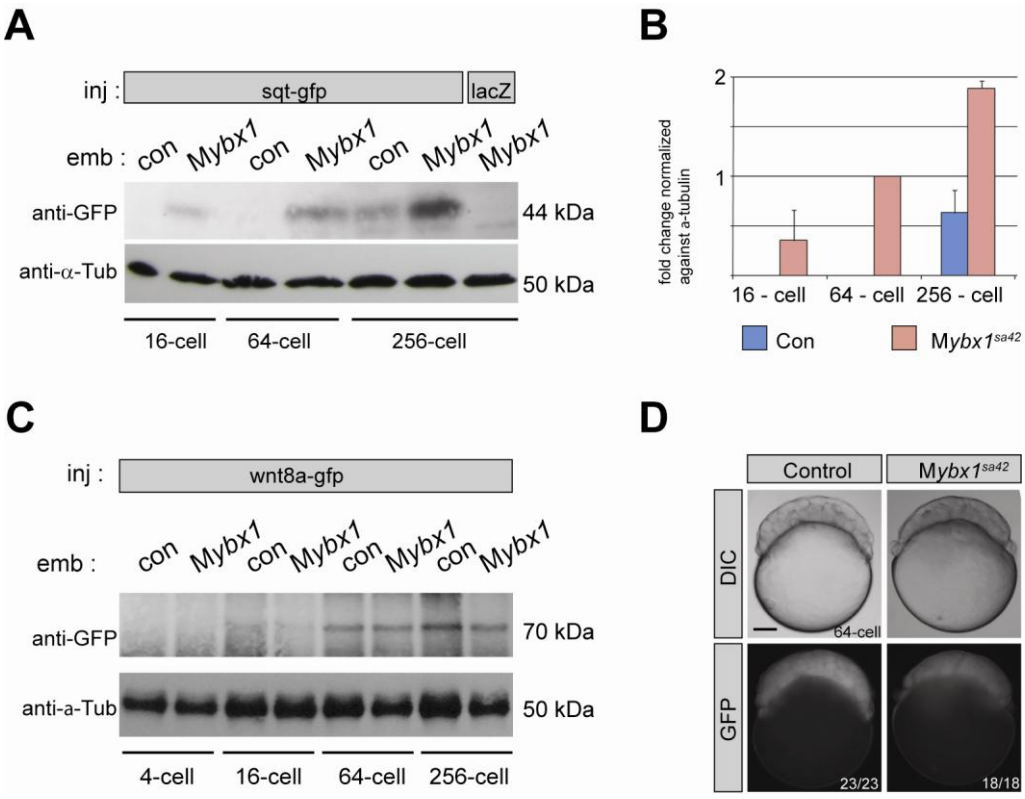

Figure 3.19 – *sqt RNA is precociously translated in Mybx1 mutant embryos.*

(A) A gfp reporter RNA when fused to sqt is translated by 16-cell stage in M*ybx1* mutant embryos whereas Sqt-GFP is detected only by 256-cell stage in control embryos. Tubulin expression is used for normalization. (B) Sqt-GFP level is elevated in M*ybx1* mutant embryos as compared to control embryos. Error bars show standard deviation from quantification of 3 independent western blot analyses. (C) A control wnt8a-gfp RNA is translated at similar rates in control and M*ybx1* mutant embryos. (D) Control gfp RNA is not translated differentially in M*ybx1* mutant embryos as compared to control embryos. GFP expression at 64-cell stage is shown by fluorescent imaging.

3.5.4 Ybx1 interacts with 5' m7G cap binding protein eIF4E

My results suggest that maternal Ybx1 is required for translational repression of maternal sqt RNA during the cleavage stages of zebrafish development. In order to understand how Ybx1 can regulate translation of sqt RNA, I examined if Ybx1 forms complexes with translation initiation factors and sqt RNA. RNA co-immunoprecipitation assays were performed using wild-type embryo extracts and antibodies against Ybx1 and translation initiation factors, eIF4E and eIF4G followed by western blot and RT-PCR to detect interactions. Ybx1 interacts with eIF4E and *vice-versa* but not with eIF4G (Figure 3.20A) while RT-PCR on immuno-precipitated samples show that sqt RNA is in complexes with Ybx1, eIF4G and eIF4E (Figure 3.20B). In contrast, gapdh and wnt8a RNA co-immunoprecipitate with the eIF4G and eIF4E proteins, but not with Ybx1. These results show that Ybx1 forms a complex with sqt RNA and 5' 7-methyl-guanosine cap binding protein eIF4E, but is not found in translation initiation complexes with other RNAs like gapdh or wnt8a (Figure 3.20B). Ybx1 has been shown to interact with the 5' cap complex and inhibit translation by displacing eIF4G (Nekrasov, 2003). These results therefore, provide evidence for a role of Ybx1 in regulation of sqt translation by binding to the translation initiation machinery and the 3'UTR of sqt RNA.

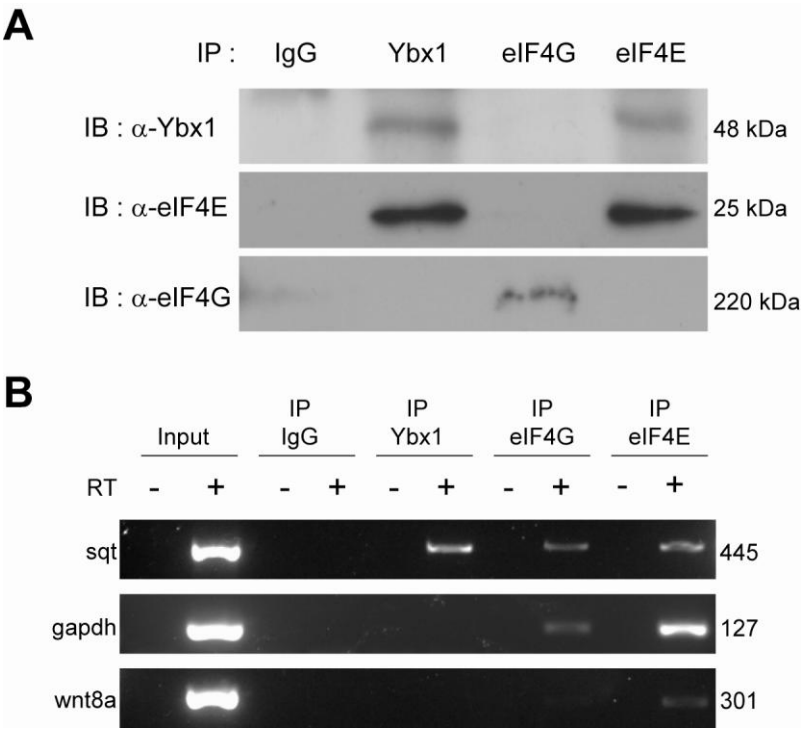

Figure 3.20 – *Ybx1 interacts with 5' m7G CAP binding protein eIF4E.*

(A) Co-immunoprecipitation assays from embryos lysates with anti-Ybx1 and anti-eIF4E antibodies followed by western blots show that Ybx1 interacts with eIF4E. eIF4G binds poorly. (B) RT-PCR on immunoprecipitated samples shows that sqt RNA forms a complex with Ybx1, eIF4E and eIF4E. Control RNAs, wnt8a and gapdh, form complex translation initiation complex proteins eIF4E and eIF4G but not with Ybx1 (band for wnt8a in eIF4G lane is very weak).

3.6 Nodal signaling is elevated in M*ybx1* embryos

3.6.1 Phosphorylation of Smad2 is precocious in M*ybx1* embryos

Sqt protein is translated prematurely in M*ybx1* mutant embryos, so I next examined when Sqt/Nodal signaling gets activated by detecting phosphorylation levels of Smad2, the downstream transducer of Nodal signaling (ten Dijke and Hill, 2004; Yeo and Whitman, 2001). I performed western blot analysis on mutant and wild-type embryo extracts at various time points during blastula stages with an antibody that specifically recognizes the phosphorylated form of Smad2. Consistent with precocious Sqt translation, I detected endogenous phosphorylated Smad2 (P-Smad2) by the 64-cell stage in M*ybx1* mutant embryos, whereas in control embryos, P-Smad2 expression is detected only at late blastula/early gastrula stages, by which time M*ybx1* mutants arrest and die (Figure 3.21A, B).

3.6.2 Expression of target genes of Nodal signaling is elevated in mutants

Since, Nodal signaling is turned on early in M*ybx1* mutant embryos I examined the induction of Nodal target genes by qPCR and RNA *in situ* hybridization. I used mutant and control embryos at 512-cell stage, before the mid-blastula transition (MBT), for qPCR analysis. Consistent with precocious and elevated phospho-Smad2 levels, expression of downstream target genes of Nodal signaling (*gsc, ntl, bon, gata5* and *sqt*) is increased in M*ybx1*mutant embryos by the 512-cell stage (Figure 3.21C). By contrast, expression of *lft2,* the Wnt target genes (*boz, vox,* and *vent*) and the FGF target genes (*spry4* and *pea3*) is either unchanged or marginally reduced in mutant embryos as compared to controls (Figure 3.21C). RNA i*n situ* hybridization, to analyze the spatial expression, shows that the YSL expression domain of *sqt* and *gsc* is expanded at the 1000-cell stage, whereas *sqt* expression is restricted to a few marginal cells in control embryos (arrowhead, Figure 3.21D) and *gsc* expression is not detected in majority of control embryos. Expression of *bon* in the presumptive endoderm is also expanded in M*ybx1* mutant embryos but not in control embryos. Expression of

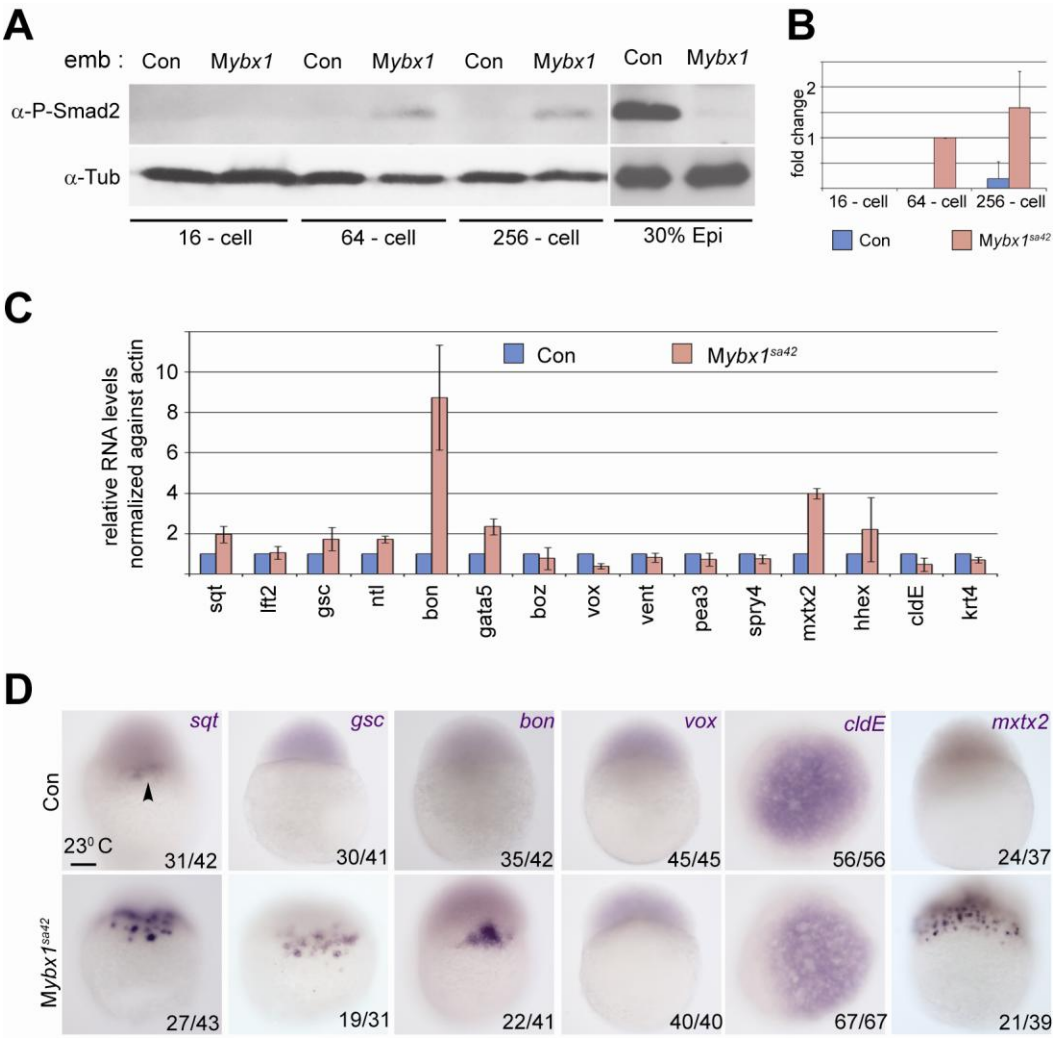

Figure 3.21 – *Nodal signaling is deregulated in Mybx1 mutant embryos.*

(A) Downstream transducer of Nodal signaling phosphorylated Smad2 (P-Smad2) is detected by 64-cell stage in M*ybx1* mutant embryos. In contrast P-Smad2 is detected only by late blastula/early gastrula stages in control embryos. Tubulin expression is used as a normalization control. 30% Epiboly lanes are from a different gel. (B) P-Smad2 level is elevated in M*ybx1* mutant embryos as compared to control embryos. (C) QPCR shows that expression of targets of Nodal signaling genes (*sqt, gsc, ntl, bon*) and YSL genes (*mxtx2, hhex1*) is elevated in M*ybx1* mutant embryos. In contrast, expression of *lefty2*, Wnt targets (*boz, vox, vent*), Fgf targets (*pea3, spry4*) and EVL genes (*cldE, krt4*) is either not affected or is slightly reduced in M*ybx1* mutant embryos. Error bars show standard deviation form 3 independent experiments. (D) RNA *in situ* hybridizations shows that *sqt, gsc* and *mxtx2* expression is expanded in the YSL of M*ybx1* mutant embryos compared to controls. Expression of *bon* in the presumptive ventral mesoderm is also expanded whereas expression of *cldE* is not affected and *vox* expression is not detected at this stage.

vox and *vent* was not detected at 1000 cell stage (*vox* in Figure 3.21D). So, taken together many Nodal target genes are expressed precociously and their levels are elevated, whereas early Wnt and FGF signaling targets are not affected in M*ybx1* mutant embryos.

3.6.3 Expression of YSL genes is also elevated in mutants

I also observed that the expression of the extra-embryonic Yolk Syncytial Layer (YSL) genes, *hhex1* and *mxtx2,* is significantly increased in M*ybx1* mutant embryos. At the same time, expression of the enveloping layer (EVL) genes, *cldE* and *krt4*, remains unaffected. *In situ* hybridizations show broader expression domain of *mxtx2* in mutants. Expression of *cldE* in the EVL in M*ybx1* mutants is comparable to that in control embryos.

3.7 The extra-embryonic YSL is expanded in M*ybx1* embryos

3.7.1 Nuclear and membrane staining show expanded YSL in mutants

To examine the YSL expansion, I labeled nuclei by DAPI staining and membranes by E-cadherin immunostaining at 1000-cell stage. Consistent with increased YSL gene expression (*mxtx2*, *hhex1*), YSL is expanded in M*ybx1* mutant embryos. Mutant embryos have several tiers of yolk syncytial nuclei (YSN) in contrast to control embryos which show only 1 tier of YSN (Figure 3.22A). E-cadherin immunostaining shows clear demarcation of membranes at the blastoderm margin in control embryos but fragmented membranes in M*ybx1* mutant embryos (higher magnification images of boxed area in bottom panel of Figure 3.22A)

3.7.2 M*ybx1* embryos show early YSL formation

To determine the timing of YSL formation, I injected SYTOX ORANGE in the yolk of embryos at 64-128 cell stage. SYTOX ORANGE is a nuclear dye that cannot penetrate live membrane and hence will label only the syncytial nuclei when YSL forms. YSN were detected as early as 256 cell stage in M*ybx1* embryos as compared to control embryos which showed YSL formation by 1000-cell stage. The number of YSL nuclei in control and mutant embryos was scored visually at 512-1000 cell stage. Approximately 50% of M*ybx1* mutant embryos show more than 13 YSN (n=59), whereas control embryos show a few or no YSN (75% with 0 nuclei, 25% <6 nuclei; n=40 embryos) (Figure 3.22B, C). The premature formation of YSL and increased numbers of YSN resulted in substantially fewer cells in the blastoderm leading to failure of gastrulation movements and eventually embryonic lethality of M*ybx1* mutant embryos by the time control embryos reach mid-gastrula stages. These phenotypes are rescued by maternal *ybx1-2a-gfp* transgene (Figure 3.22B, C). Nearly 80% of M*ybx1* embryos with P*Tg*, show 7 or more YSN and ~25% show >20 YSL nuclei whereas M*ybx1* embryos with M*Tg* (75% show no YSN, 25% show <7 YSN) show normal numbers of YSL nuclei. Thus, the extra-embryonic YSL forms precociously and is expanded in M*ybx1* mutant embryos.

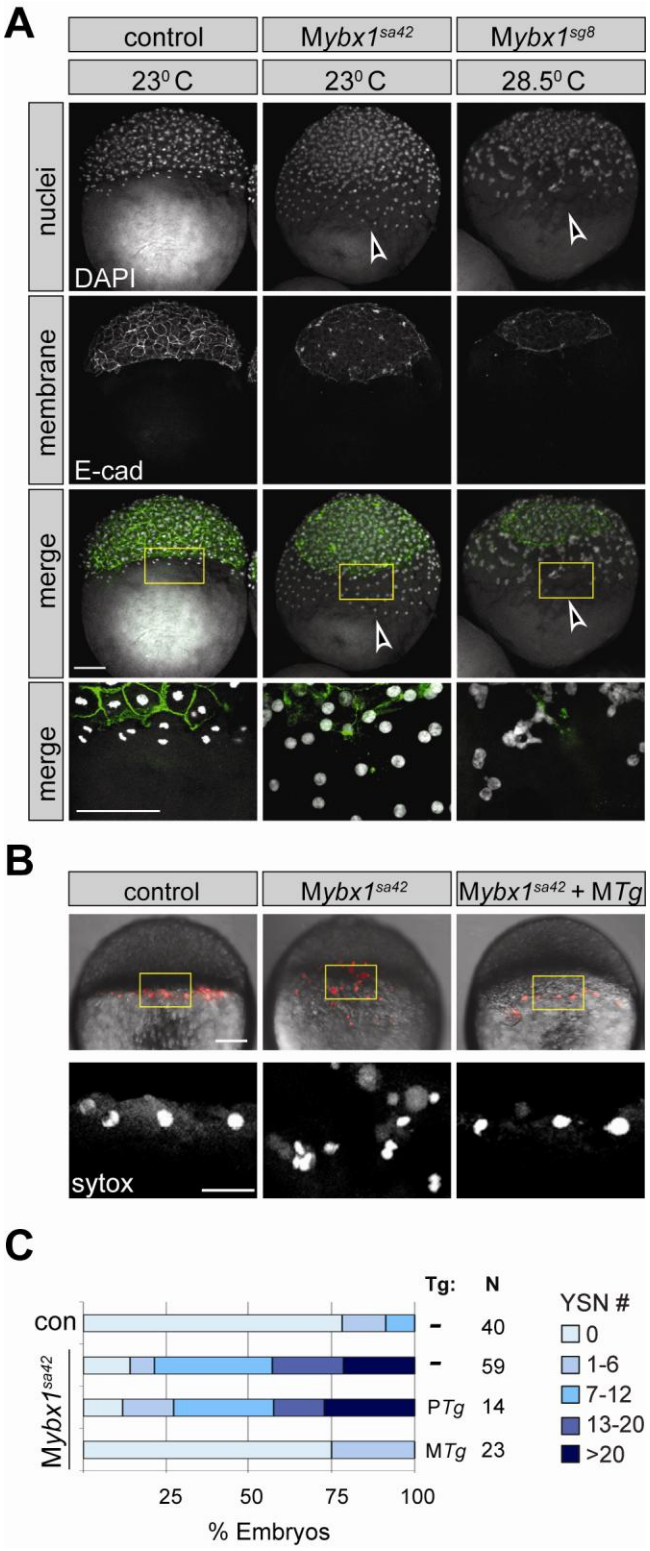
A
control
Mybx1sa42
Mybx1sg8
23⁰ C
23⁰ C
28.5⁰ C
nuclei
DAPI
membrane
E-cad
merge
merge
B
control
Mybx1sa42
Mybx1sa42 + MTg
sytox
C
Tg:
N
con
-
40
Mybx1sa42
-
59
PTg
14
MTg
23
YSN #
0
1-6
7-12
13-20
>20
25
50
75
100
% Embryos

Figure 3.22 – *Mybx1 mutant embryos have expanded extra-embryonic YSL.*

(A) DAPI staining to label nuclei and E-cadherin immunostaining to detect membranes shows 1 tier of YSL nuclei (YSN) in control embryos. In contrast, M*ybx1*^{sa42} and M*ybx1*^{sg8} mutant embryos have several layers of YSN (arrowheads). Yellow boxed areas in merge panel are showed at higher magnification in the bottom panels. Cell membrane forms clear boundary in control embryos but appears fragmented in M*ybx1* mutant embryos. (B) Sytox orange injection in yolk shows multiple layers of YSN in M*ybx1* mutants as compared to controls. Sytox orange was injected at 64-128 cell-stages and number of YSN was scored at 512-1000 cell stage. Scale bars, 100 µm. (C) Histograms showing numbers of YSN in control and M*ybx1*^{sa42} mutant embryos, with or without *ybx1* transgenes. Number of embryos scored is indicated on the right.

3.8 Nodal diffusion from yolk leads to expanded YSL

3.8.1 Wild type embryos implanted with Nodal beads show more YSL nuclei

Ybx1 is a multi-functional protein that regulates gene expression of several target genes at both transcriptional and translational levels (Eliseeva et al., 2012; Kohno et al., 2003). This raises a question, whether the phenotypes observed in M*ybx1* mutants are a direct consequence of deregulated Nodal/Sqt signaling from the yolk to the blastoderm, or due to other effects of Ybx1. To directly determine the effects of excess Nodal protein from the yolk on embryogenesis, we implanted affi-gel beads that were pre-soaked in either control BSA protein or purified mouse Nodal protein, into the yolk of wild-type embryos at the 32-cell stage, and examined YSL nuclei at 1000-cell stage (Figure 3.23A). Bead implantation procedure does not affect embryonic patterning as BSA bead-implanted embryos appear morphologically normal and develop similar to non-manipulated embryos (Figure 3.23A). Nuclear staining shows control BSA bead-implanted embryos have 1 tier of YSL (n=17), similar to wild-type embryos (Kimmel and Law, 1985). By contrast, the majority of Nodal bead-implanted embryos have more YSN (75%, n=32 embryos; Figure 3.23B, C). Taken together, these results suggest that Nodal protein diffusing from the yolk is sufficient to induce YSL fate and increase the number of YSL nuclei.

3.8.2 Nodal bead implantation in MZ*oep* embryos does not lead to more YSN

We performed Nodal bead implantation in the yolk of MZ*oep* mutant embryos, which are unable to respond to Nodal signals (Gritsman et al., 1999). Interestingly, bead implanted MZ*oep* embryos do not show more YSN (n=13, Figure 3.23B, C). This further supports our finding that Nodal signaling from the yolk can induce premature and expanded extra-embryonic YSL.

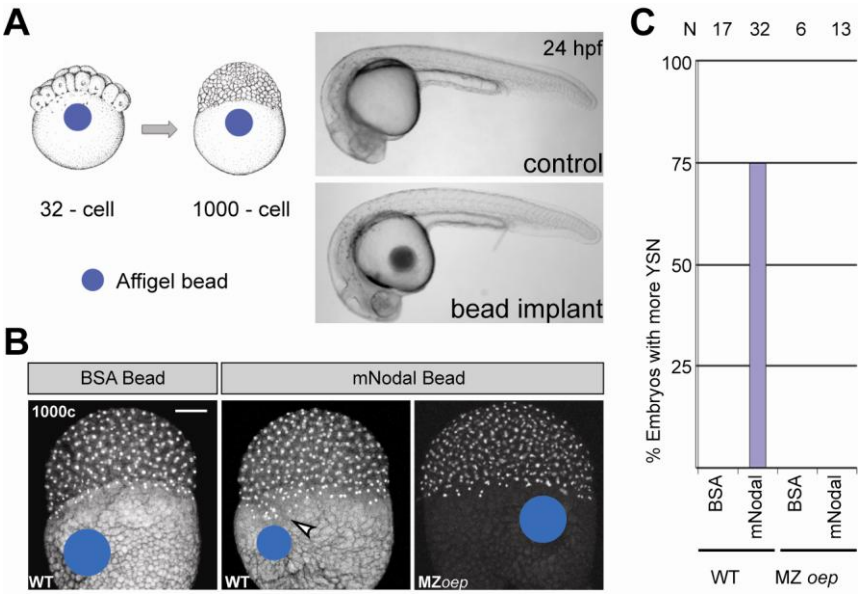

Figure 3.23 – *Nodal bead implantation in the yolk results in more YSL nuclei.*

(A) Schematics to show the design of bead implantation experiment. Control BSA or mouse Nodal coated beads were implanted in 32-cell stage wild-type embryos and YSL nuclei were examined at 1000 cell-stage. Bead implantation did not affect the morphology of embryos as seen at 24hpf. (B) DAPI staining shows one tier of YSN in BSA bead implanted embryos whereas mNodal bead implanted embryos show many YSL nuclei (arrowhead). MZ*oep* embryos do not show extra YSN upon mNodal bead implantation. Blue circle indicates the position of implanted bead. Scale bar, 100µm. (C) Histogram showing percent of wild-type or MZ*oep* embryos with more YSN after bead implantation. Number of embryos examined is indicated on top of the histograms.

3.9 Blocking Sqt/Nodal Signaling can rescue gastrulation arrest in M*ybx1* embryos

Our results suggest that the phenotypes observed in M*ybx1* embryos are a result of precocious and elevated Nodal signaling. Hence, in order to rescue these phenotypes, we decided to block Nodal signaling by two means – a) by overexpression of Nodal inhibitor, Lefty1 (Lft1) and b) by generating *ybx1;sqt* compound mutants.

3.9.1 Overexpression of Lft1 can rescue gastrulation arrest in M*ybx1* embryos

In order to block excess Nodal signaling in M*ybx1* embryos, I overexpressed Nodal inhibitor, Lft1 by capped RNA injection. Lft1 is a bona-fide Nodal inhibitor which functions either by binding to the Nodal ligands or the co-receptor (Chen and Shen, 2004). Nuclear and membrane staining of M*ybx1* mutant embryos injected with lft1 RNA shows that lft1 overexpression restores the membrane structure and rescues the YSL expansion (Figure 3.24A). M*ybx1* mutant embryos injected with lacZ RNA show several tiers of YSN but mutant embryos injected with lft1 show 1 tier of YSN similar to the control embryos injected with either lacZ or lft1 RNA (Figure 3.24A). Control and mutant injected embryos were scored for gastrulation and survival. A significant number of M*ybx1* mutant embryos (~60%, N= 299) injected with lft1 RNA initiated gastrulation movements and nearly 80% of them survived till prim5 stage in contrast to lacZ injected M*ybx1* mutant embryos (Figure 3.24B). All lefty injected embryos exhibited a range of lefty overexpression phenotypes at prim5 stage showing the efficacy of lft1 overexpression.

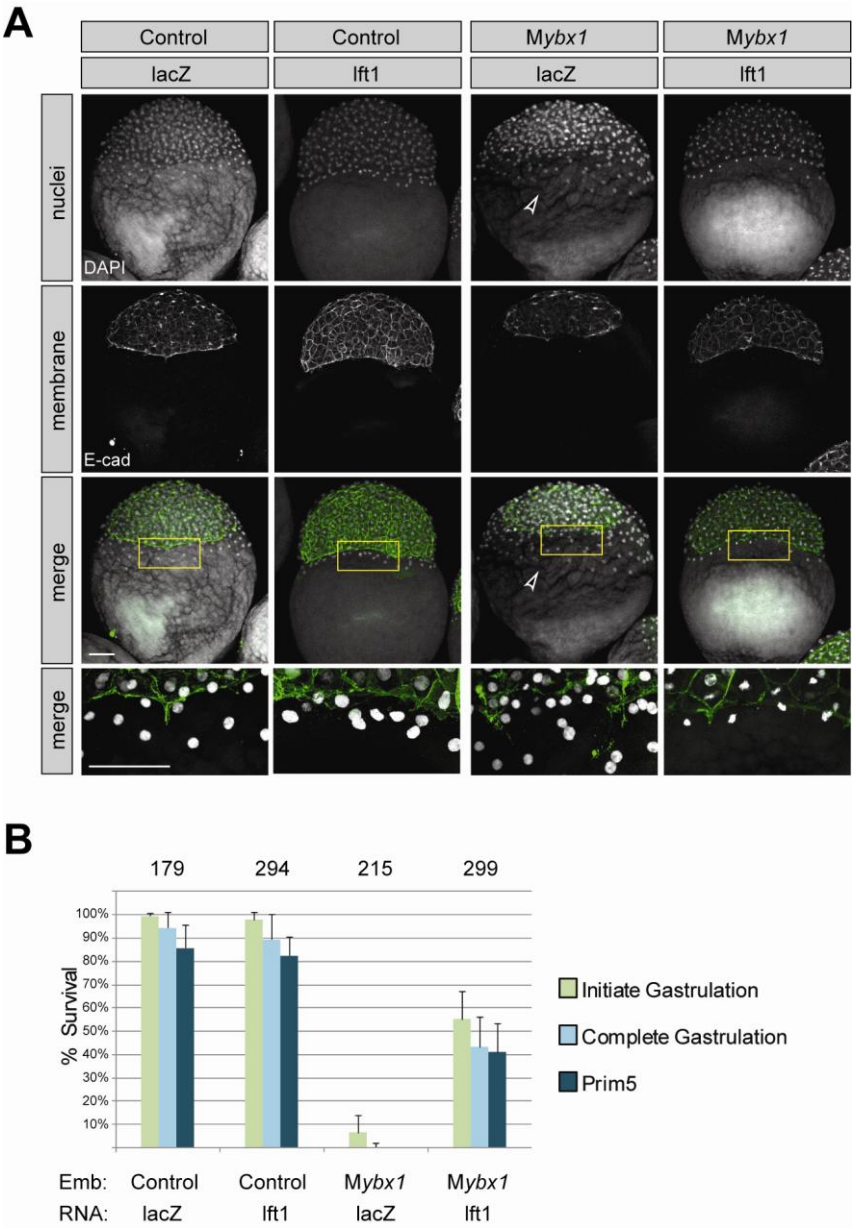

Figure 3.24 – *Lefty1 overexpression restores YSL expansion and gastrulation defects in Mybx1 mutant embryos.*

(A) DAPI staining to label nuclei and E-cadherin immunostaining to mark membranes show that lefty1 injected M*ybx1* mutant embryos exhibit normal YSL formation with 1 tier of YSL nuclei similar to lacZ or lefty1 injected control embryos. lacZ injected M*ybx1*mutant embryos show expanded YSL formation. Yellow boxed area in merge panel is shown at higher magnification in the bottom panels. Scale bar, 100µm. (B) Histogram showing percentage of embryos that initiate and complete gastrulation and survive till prim5 when subjected to temperature shift at 23^{o} C. Overexpression of lefty1 but not lacZ leads to rescue of gastrulation defects in M*ybx1* mutant embryos. Number of embryos scored is shown on top of the histogram. Error bars show standard deviation from 3 experiments.

3.9.2 YSL and gastrulation defects in M*ybx1* mutant embryos are rescued in M*ybx1*;*sqt* compound mutants

To block Nodal signaling in M*ybx1* embryos we also generated *ybx1;sqt* compound mutants. The mutant allele sqt^{cz35} is a spontaneous insertion in *sqt* intron1 which leads to truncated Sqt protein that is not functional (Bennett et al., 2007; Feldman et al., 1998) but the mutant RNA is expressed and localized similar to wild-type sqt RNA (Lim et al., 2012). Thus sqt^{cz35}mutation leads to lack of Sqt signaling without affecting the non-coding function of sqt RNA. I screened >200 fishes but did not recover any *ybx1;sqt* double homozygous adult fish. So, we examined embryos from $ybx1^{sa42/sa42};sqt^{cz35/+}$ crosses which will yield 25% M$ybx1^{sa42};sqt^{+/+}$ (reduced maternal Sqt), 50% M$ybx1^{sa42};sqt^{cz35/+}$ (reduced maternal and zygotic Sqt) and 25% M$ybx1^{sa42};sqt^{cz35/cz35}$ (reduced maternal and no zygotic Sqt) embryos. Interestingly, ~80% of embryos from $ybx1^{sa42/sa42};sqt^{cz35/+}$ crosses completed gastrulation and survived till prim5 stage unlike M*ybx1* single mutants (Figure 3.25A). Genotyping the surviving embryos show that nearly all M*ybx1;*Z$sqt^{cz35/cz35}$ embryos (25.6%, Figure 3.35B) survive whereas some embryos with either one or both copies of wild-type *sqt* fail to gastrulate normally and eventually die. M*ybx1;*Z$sqt^{cz35/cz35}$ compound mutants show phenotypes typical of reduced Nodal activity such as those observed in MZ*midway* mutant embryos, or complete loss of Nodal activity (Figure 3.25C) (Schier, 2009a; Slagle et al., 2011; Thisse et al., 2000). In conclusion, these results suggest that YSL and gastrulation defects observed in M*ybx1* mutant embryos are a direct consequence of deregulated Sqt/Nodal signaling because of the absence of Ybx1 function and can be rescued by blocking Sqt/Nodal signaling.

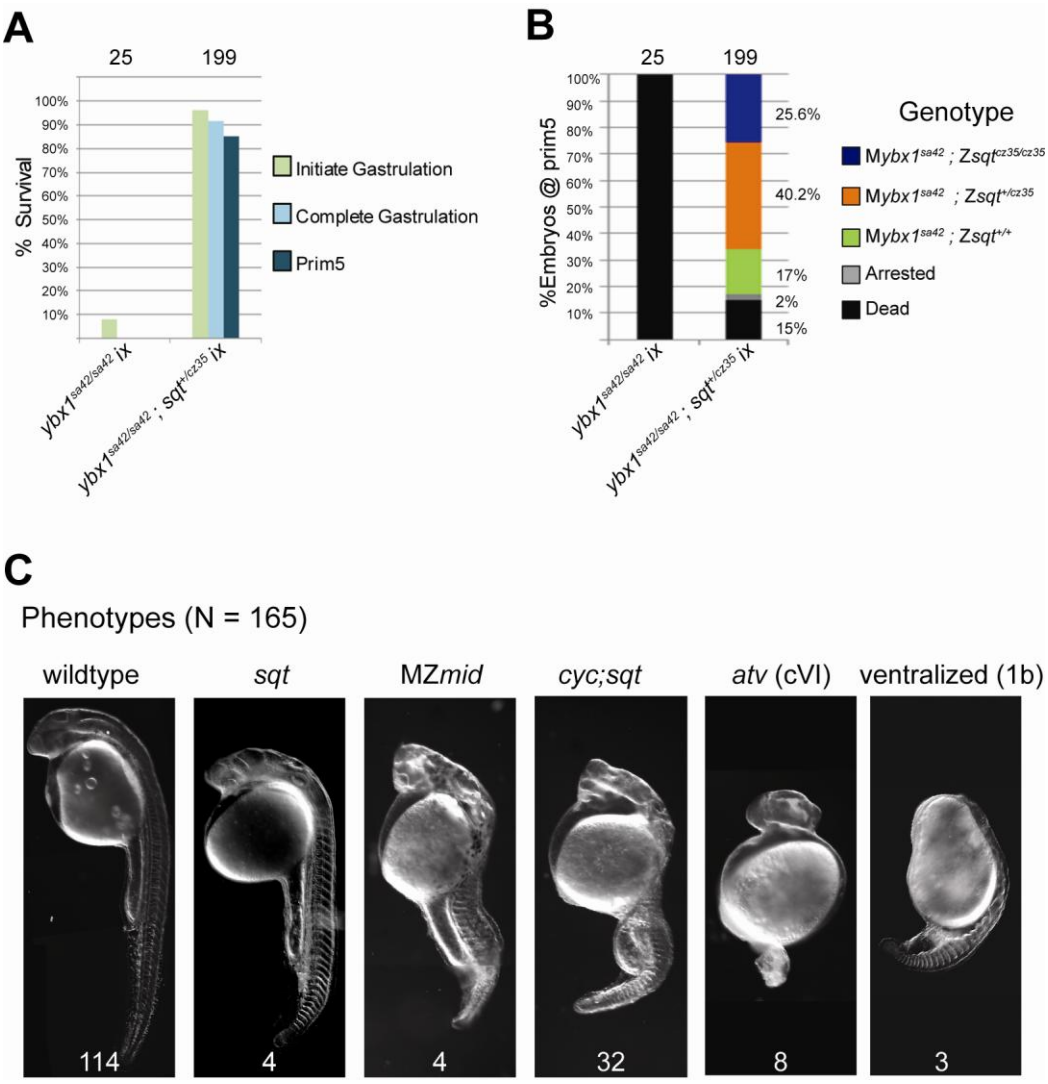

Figure 3.25 – *YSL and gastrulation defects in Mybx1 embryos are rescued by blocking Nodal signaling.*

(A) Histogram showing percentage of embryos that initiate and complete gastrulation and survive till prim5 stage when subjected to temperature shift at 23° C. Most embryos from *ybx1*$^{sa42/sa2}$; *sqt*$^{cz35/+}$ crosses initiate and complete gastrulation in comparison to *ybx1*$^{sa42/sa42}$ crosses. (B) Histogram showing the genotypes of embryos those survive till prim5 stage when subjected to temperature shift. The expected % for each genotype is 25% for M*ybx1*^{sa42};*sqt*$^{cz35/cz35}$ and M*ybx1*^{sa42};*sqt*$^{+/+}$, and 50% for M*ybx1*^{sa42};*sqt*$^{cz35/+}$. All M*ybx1*^{sa42};*sqt*$^{cz35/cz35}$ embryos (which have no Sqt signaling) survive, whereas many M*ybx1*^{sa42};*sqt*$^{cz35/+}$ and M*ybx1*^{sa42};*sqt*$^{+/+}$ do not survive at 23°C. Number of embryos scored is indicated above the histogram, and % observed for each genotype is indicated at the right. (B) DIC images of prim-5 stage embryos from *ybx1*$^{sa42/sa42}$;*sqt*$^{cz35/+}$ crosses show varying *nodal* phenotypes. Phenotypes were scored as wild type, *squint*, MZ*midway*, *cyc;sqt*, antivin/lefty overexpression class VI, and ventralized *ichabod* 1b-like. The number of embryos scored for each class is at the bottom of each image.

Chapter 4

Discussion

4.1 Post transcriptional regulation of maternal sqt RNA

During oocyte maturation, transcripts are produced and may be reversibly silenced. Embryos of most animals transcribe only after the zygote divides one or more times. In zebrafish, the maternal to zygotic transition (Mid Blastula Transition, MBT) takes place at the 10^{th} cleavage of the developing blastula by when a number of patterning and cell fate specification events have already taken place (Abrams and Mullins, 2009; Dosch et al., 2004; Kane and Kimmel, 1993; Wagner et al., 2004). Hence, post-transcriptional regulation of maternally deposited mRNAs plays a crucial role in embryonic patterning. Maternal RNAs are subjected to various levels of regulations like spatial localization, RNA stability, regulated processing of pre-mRNA and translational regulation (Bashirullah et al., 2001; Bettegowda and Smith, 2007; Duval et al., 1990; Johnstone and Lasko, 2001; Kloc and Etkin, 2005; Martin and Ephrussi, 2009; Meric et al., 1996; Pepling, 2010; Slater et al., 1973). We found that maternal sqt RNA is spatially restricted to 2 cells in the 4-cell stage embryos and this event of localization predicts future dorsal of the developing zebrafish embryos (Gore et al., 2005). In this study, we discovered how several aspects of post-transcriptional regulation of sqt RNA are critical for zebrafish embryogenesis.

A ~20 bp motif (DLE) consisting of both sequence and structural information in the sqt 3'UTR confers dorsal localization to sqt transcripts (Gilligan et al., 2011). An AGCAC motif contributes most significantly to sqt RNA localization. This motif is similar to vegetal localization element UUCAC, identified from RNAs localized to the vegetal pole in *Xenopus* oocytes and found to be conserved in other chordates as well (Betley et al., 2002; King et al., 2005), and germplasm localization motif, GCAC (Chang et al., 2004; Choo et al., 2005). However, sqt RNA is seen neither localized to the vegetal pole nor to the germplasm (Sampath Lab unpublished observations). This suggests that the CAC motif in sqt is different for germline and vegetal RNAs. Alternatively, the additional stem-loop structure in DLE may act in a combinatorial manner (Betley et al., 2002) with the AGCAC motif and provide specificity to dorsal localization of sqt RNA. Furthermore, the ATG region of sqt RNA is also essential for dorsal

localization. Our results suggest an interaction between the DLE in 3'UTR and the ATG region in 5'UTR. Hence, it is conceivable that sqt RNA is circularized in the localizing RNP and various elements in the UTRs may bind to distinct components of the localizing machinery, which may function in different steps of localization. In support of the above possibility, sqt ATG targeting morpholinos (sqt MO1) and DLE targeting morpholinos (DLE MO) affect localization of sqt RNA in significantly different manners. Sqt MO1 when co-injected with fluorescent sqt RNA results in aggregates in yolk in >30% embryos whereas DLE MO co-injections lead to aggregates in yolk in only ~16% embryos (Gilligan et al., 2011). Hence the ATG region contributes significantly to the yolk to blastoderm translocation of sqt RNA. Such bipartite signals for distinct steps in RNA localization have been uncovered in the context of gurken RNA in *Drosophila* oocytes and *ASH1* RNA in budding yeast. (Gonzalez et al., 1999; Thio et al., 2000). Elements in the gurken 5'UTR are required for its oocyte localization during early stages of oogenesis while elements in the gurken 3' UTR confer tight antero-dorsal localization during late stages of oogenesis. Translation dependent localization and anchoring of RNAs also rely on multiple *cis*-elements like in yeast *ASH1* and *Drosophila* oskar and gurken RNAs (Gonzalez et al., 1999; Gunkel et al., 1998; Saunders and Cohen, 1999). Finally, full-length sqt RNA always localizes more efficiently as compared to heterologous sequences fused to the sqt 3'UTR, suggesting the presence of some additional elements in the coding sequence.

In this study, we purified Ybx1 as the DLE binding protein and provide several lines of evidence to show that Ybx1 functions as a core component of post-transcriptional regulation of sqt RNA. Maternal Ybx1 is essential for sqt localization and is also required for regulated processing and translation of sqt transcripts.

4.2 Biochemical purification of DLE binding factor, Ybx1

Purification of RNA binding proteins (RBPs) is a critical step for studying RNA metabolism. Commonly used methods for identifying RBPs are – 1) Screening protein libraries for binding to a specific RNA, 2) A series of chromatographic fractionations (or separation of proteins by electrophoresis) coupled with an RNA binding assay for identification, 3) Affinity based purification methods to isolate multi-protein complexes (Hegarat et al., 2008). While screening of protein libraries is an ideal method for identification of proteins expressed in limiting amounts, the *in vitro* nature of the method may result in either false positives or false negatives. Affinity based methods are commonly accomplished by labeling RNA with small molecules like Biotin (Scaturrok et al., 2003) or RNA aptamers that can bind to small molecules/proteins such as streptomycin (Windbichler and Schroeder, 2006); tobramycin (Hartmuth et al., 2004);*Pseudomonas phage 7*, PP7 (Hogg and Collins, 2007); MS2 coat protein (Slobodin and Gerst, 2010), polypyrimidine tract binding protein, PTB (Sharma, 2008); iron responsive element (IRE) binding protein (Rouault et al., 1989). These small molecules/proteins in turn can be immobilized to prepare the affinity matrix. Affinity purification results in the isolation of a large number of candidates and validation experiments are time consuming. Furthermore, the nature of interactions in this case can be direct or indirect. In contrast, chromatographic fractionation leads to partial purification of direct RNA binders. Mass-spectrometry of the partially purified sample results in relatively fewer candidates that can be easily validated by RNA gel-shifts, UV-crosslinking and RNA immunoprecipitation experiments. Nonetheless, all these methods rely on good quality protein lysate preparation in adequate amounts, especially if the candidate protein is limiting in nature. As zebrafish exhibits high fecundity, embryos laid by females are an excellent source of protein lysates. Overexpression of non-native proteins in embryos is also relatively easy via RNA injections.

Our principal aim was to identify the proteins that directly bind to specific elements in the sqt 3'UTR, so we purified sqt RNA binding factors (SRBFs) by

chromatographic fractionation of zebrafish embryo lysates followed by RNA gel shifts. At the next level, affinity purification techniques can be used to pull down components of the sqt RNP. The sqt DLE binding factor, SRBF1 was identified as Ybx1. Ybx1 is a multifunctional protein having roles in many contexts. Hence zebrafish embryos can serve as a system to purify core components of localization and translational regulation complexes that may be relevant in other cell types such as neurons, germ-cells, polarized epithelia etc. For example, Staufen and IGF II – mRNA binding protein (Imp) are involved in RNA localization in oocytes as well as neurons (Boylan et al., 2008; Roegiers and Jan, 2000).

4.3 Ybx1 – A multifunctional protein

Ybx1 is a multifunctional DNA-RNA binding protein with roles in DNA repair and replication, transcription, RNA transport, pre-mRNA splicing and translation (Eliseeva et al., 2012; Kohno et al., 2003; Wolffe, 1994). Ybx1 is a member of a large family of proteins with an evolutionary conserved cold-shock domain. The ascidian Ybx1 homolog, CiYB1 is found to be a core component of messenger ribonucleoprotein (mRNP) particles in gonads. CiYB1 is found in complexes with posteriorly localized RNAs Cipem and Ci-macho1 and is involved in their translational regulation in C*iona* embryos (Tanaka et al., 2004). The *Drosophila* Ybx1 homolog, Yps forms complex with Exu and *oskar* RNA during its localization, and also interacts with Cup and eIF4E in the translational regulation complex (Mansfield et al., 2002; Wilhelm et al., 2003; Wilhelm et al., 2000). *Xenopus* Ybx1 homologs, FRGY1 and FRGY2, specifically recognize the AACAUC sequence motif in RNA via the cold shock domain (Bouvet et al., 1995). FRGY proteins are also considered as a major component of storage mRNA particles in *Xenopus* oocytes and function by masking maternal RNAs (Marello et al., 1992; Murray et al., 1991). The Ybx1 knock-out mouse is embryonic lethal with defects in neural tube formation and cell proliferation (Uchiumi et al., 2006). The Ybx1 homolog in humans, YB1 functions widely as a translational regulator in epithelial to mesenchymal transition (EMT) and metastatic progression (Evdokimova et al., 2009a; Evdokimova et al., 2009b;

Mouneimne and Brugge, 2009). In addition, Ybx1 is also present in neuronal RNP complexes. Ybx1 binds to GluR2 and CaM1 RNAs in neuronal cells and regulate their translation in an activity dependent manner (Tanaka et al., 2010; Tanaka et al., 2012). Furthermore, Ybx1 is associated with Staufen containing mRNPs in neuronal dendrites (Maher-Laporte et al., 2010). Fragile X Mental Retardation protein (FMRP), in neuronal mRNP particles, possibly functions in translational modulation by interacting with Ybx1 (Ceman et al., 2000).

4.4 Specificity of sqt-Ybx1 interaction

Zebrafish Ybx1 is maternally expressed and both RNA and protein are uniformly distributed during early zebrafish embryogenesis. Ybx1 binds to nucleic acids in various contexts and leads to multiple downstream effects (Eliseeva et al., 2012; Kohno et al., 2003). This raises the question of how specificity is achieved in the sqt-Ybx1 interaction. In early zebrafish embryos, Ybx1 binds to sqt 3'UTR in a sequence specific manner as antisense sqt 3'UTR, vg1 3'UTR and wnt8a 3'UTR do not interact with Ybx1. Competition binding assays with excess of control RNAs also show the specificity of sqt-Ybx1 interaction.

4.4.1 Modular design of RNA binding proteins confer specificity

We found that the CSD and adjacent domains (ssDBD, RNP, DD) of Ybx1 are required for sqt binding whereas the C-terminal half is dispensable. Ybx1 CSD has been shown to bind to specific sequence motifs in RNA while the C-Terminal domain binds to RNA in a non-sequence specific manner (Bouvet et al., 1995; Coles et al., 2004; Izumi et al., 2001; Nekrasov et al., 2003a; Swamynathan et al., 2000). Hence, it is possible that the CSD and other nucleic acid binding domains in Ybx1 function cooperatively and confer specificity. For example, another CSD containing protein Lin28A regulates biogenesis of let-7 RNA by binding to two distinct regions via a bipartite RNA recognition module consisting of two folded domains (Nam et al., 2011). Fragile X mental retardation

protein (FMRP) also utilizes two distinct RNA-binding domains to bind to distinct elements in the target RNA (Ascano et al., 2012).

4.4.2 Components of the ternary complex provide specificity

Genome wide studies in yeast, *C.elegans* and HeLa cells show that RNA binding proteins (RBPs) can bind to several target mRNAs (Campbell et al., 2012a; Castello et al., 2012; Hieronymus and Silver, 2003; Hogan et al., 2008). However, the protein architecture of RBPs consisting of a modular design provides context dependent specificity (Castello et al., 2012). Specificity in RNA-protein interaction is also conferred by other proteins in the ternary complex. For example, Cytoplasmic Polyadenylation Element Binding (CPEB) protein, CPB-1 and PUF protein FBF-2 function cooperatively in translational repression (Campbell et al., 2012b). Deep sequencing analysis of RNA bound to these proteins shows that a difference in RNA binding specificity is induced by interaction between CPB-1 and FBF-2 (Campbell et al., 2012a). A similar mechanism has been uncovered in the context of *ASH-1* RNA localization in yeast. A complex of RBPs, She2p and She3p function synergistically and show a higher binding affinity for the localizing RNA, *ASH-1*, as compared to any control RNA. However, none of the individual proteins show highly specific cargo binding (Muller et al., 2011). In accordance with this we found that RNA gel-shift with rYbx1 runs faster than the endogenous SRBF1 shift, suggesting that the endogenous shift might have other proteins in the complex with Ybx1. Hence, specificity in RNA-protein interaction can be conferred by modular design of RBPs and formation of a ternary complex where other proteins provide context-specific binding.

4.5 Conditional disruption of Ybx1

Sometimes, a gene can have distinct roles during different stages of development and a null mutant will only enable us to study the earliest function. The use of conditional mutants can be useful in analyzing maternal effect genes which have essential functions in zygotic development. Since Ybx1 is an abundant molecule with several functions so a complete knock-out of *ybx1* may be lethal or will affect multiple pathways. Therefore, our study was facilitated by the use of a temperature sensitive *ybx1* allele, *ybx1*^{sa42}. By conditional disruption of maternal Ybx1 at specific time-points, we uncovered a role of maternal Ybx1 in regulation of Nodal signaling during blastula stages of zebrafish development. Hence this allele can be potentially used to identify other targets of Ybx1 at different stages of developments and cellular processes regulated by them.

4.6 Functions of Ybx1 in sqt RNA localization, processing and translation

My work discovered that a major function of maternal Ybx1 is to regulate Nodal signaling by participating in sqt RNA localization, processing, and translation. Consistent with Ybx1 being a sqt-DLE binding protein, localization of sqt RNA is severely disrupted in M*ybx1* mutant embryos. RNAs localized in zebrafish oocytes/early embryos are broadly classified in four categories (ubiquitous, animal, vegetal and cortical) (Howley and Ho, 2000). I examined spatial distribution of several RNAs from these categories in early embryos and found that only sqt RNA localization is disrupted in M*ybx1* mutant embryos.

In zebrafish embryos, maternally deposited sqt RNA is majorly unspliced and lacks mature polyA tail. Spliced and polyadenylated sqt can only be detected by the 16-cell stage and afterwards (Aanes et al., 2011; Gore, 2007; Lim et al., 2012). Interestingly, sqt RNA is precociously spliced and polyadenylated in M*ybx1* mutant embryos. Splicing dependent localization has been observed in the case of oskar RNA in *Drosophila* where assembly of the exon-junction complex (EJC) is required for localization (Ghosh et al., 2012; Hachet and Ephrussi, 2004).

Studies in both mouse and *Drosophila* cells suggest that Muscleblind proteins, implicated in myotonic dystrophy, localize specific mRNA isoforms after regulated splicing (Wang et al., 2012). *In vitro* synthesized sqt RNA that lacks both introns localizes similar to the endogenous sqt (Gore et al., 2005). This suggests that either introns are not absolutely required for localization or that injected RNA forms a complex with endogenous RNA and gets included in the localizing RNP. To understand how the events of localization and splicing are linked in the context of sqt RNA, and how Ybx1 regulates splicing, further experiments are required. Ybx1 has been identified in human spliceosomal complexes (Deckert et al., 2006) and also been shown to regulate splice site selection by interacting with the splicing factor Srp30c and binding to splicing recognition motifs (Raffetseder et al., 2003). But the field of cytoplasmic splicing is relatively new and controversial and needs further work (Konig et al., 2007; Steitz et al., 2008). The Ybx1-sqt RNA interaction can serve as a good model for studying splicing segregation and minor spliceosomes outside the nucleus.

The polyA tail at the end of 3'UTR of RNAs plays an important role in their translatability and regulating the length of the poly-A tail is a common means of translational regulation of maternally deposited RNAs prior to fertilization (Meric et al., 1996). Partially adenylated mRNAs are stored in the cytoplasm of sea urchin oocytes and the length of poly-A increases by more than 2-folds after fertilization (Slater et al., 1972). In *Xenopus* oocytes, many cytoplasmic polyadenylation element (CPE) containing RNAs like cyclin B1 and gld-2 are actively deadenylated by a PUF family protein Pumilio and held in a translational repressed state (Radford et al., 2008; Simon et al., 1992). Several translational repressor proteins including Pumilio and Nanos recruit the conserved deadenylase complex CCR4-Pop2-Not (Goldstrohm et al., 2006; Kadyrova et al., 2007). After fertilization, maternal RNAs undergo cytoplasmic polyadenylation and become translationally active (Slater et al., 1972; Slater et al., 1973). Hence, mutations affecting the poly-A dependent activation of masked maternal RNAs lead to developmental arrest (Lieberfarb et al., 1996). Ybx1 or other partner proteins in the sqt RNP granule may deadenylate maternal sqt RNA or actively

inhibit 3'-end processing and polyadenylation like an interacting protein PTB (Polypyrimidine tract binding protein (Castelo-Branco et al., 2004; Cobbold et al., 2010)). Hence, in M*ybx1* mutant embryos, sqt is polyadenylated much earlier.

In M*ybx1* mutant embryos, sqt translation is also deregulated. Our localization studies suggested that DLE in the sqt 3'UTR and ATG region in the 5'UTR of sqt RNA interact with each other. Hence, 3'UTR binding proteins might interact with the 5' 7-methyl-guanosine cap complex or the ribosomal complex in 5'UTR. Such interactions have been shown in context of translational control of maternal mRNAs in *Xenopus* oocytes where CPEB interacts with eIF4E via Maskin. Maskin binds to cap binding protein eIF4E, and blocks association between eIF4G and eIF4E, hence represses translation by preventing recruitment of the 40S ribosome subunit to the 5'end of mRNAs (Cao and Richter, 2002). A similar mechanism is observed in the regulation of oskar RNA translation in *Drosophila* oocytes, where 3'UTR binding protein Bruno interacts with eIF4E binding protein Cup (Nakamura et al., 2004b). The Ybx1 homolog, Yps is also present in the oskar RNP complex and immunoprecipitates with Cup and eIF4E (Wilhelm et al., 2003). Mammalian YB1, is also known to prevent eIF4G from binding to eIF4E, and blocks initiation of translation (Nekrasov et al., 2003b). Binding of Ybx1 to the sqt 3'UTR and eIF4E in zebrafish embryos likely prevents eIF4G-eIF4E complex formation and hence blocks translation. In M*ybx1* mutants, Sqt translation occurs precociously, suggesting that the binding of Ybx1 to the translation initiation factors and the sqt 3'UTR can lead to translational repression of sqt RNA.

4.7 Sqt/Nodal signaling and YSL expansion

Premature translation of Sqt in M*ybx1* mutant embryos leads to deregulated Sqt/Nodal signaling and many Sqt/Nodal target genes are precociously induced and their expression domains are expanded in YSL. Surprisingly, *lefty2* expression is not induced in M*ybx1* mutant embryos although *lefty* genes are Nodal targets (Branford and Yost, 2002; Feldman et al., 2002; Meno et al., 1997). Thus, initiation of *lefty2* expression may require some other factors that are not induced in M*ybx1* mutant embryos. Consequently, the synergistic effect of elevated Sqt protein and lack of feedback inhibition by Lefty2 likely aggravates deregulated Nodal signaling in M*ybx1* mutant embryos. Subsequently, the extra-embryonic YSL fate is expanded in M*ybx1* mutant embryos leading to gastrulation failure. The expanded YSL and gastrulation defects observed in M*ybx1* mutant embryos were also reported in lefty-1,lefty-2 double morphant embryos, where Nodal signaling is deregulated in the absence of the Lefty inhibitors (Feldman et al., 2002). In Nodal bead implantation experiments, implants in MZ*oep* embryos lacking Nodal signaling (Gritsman et al., 1999) do not lead to more YSL nuclei. Furthermore, YSL and gastrulation defects in M*ybx1* mutant embryos can be rescued by blocking Nodal signaling by lefty overexpression or by using the sqt^{cz35} genetic background that lacks the signaling functions of Sqt. Taken together, phenotypes observed in M*ybx1* mutant embryos are the consequence of excess Nodal signaling (Figure 4.1). Interestingly, M*ybx1;*Z*sqt* compound mutant embryos exhibit phenotypes similar to *cyc;sqt* double mutants (Feldman et al., 1998) instead of *sqt* mutants, suggesting that maternal Ybx1 may regulate Nodal signaling at additional steps.

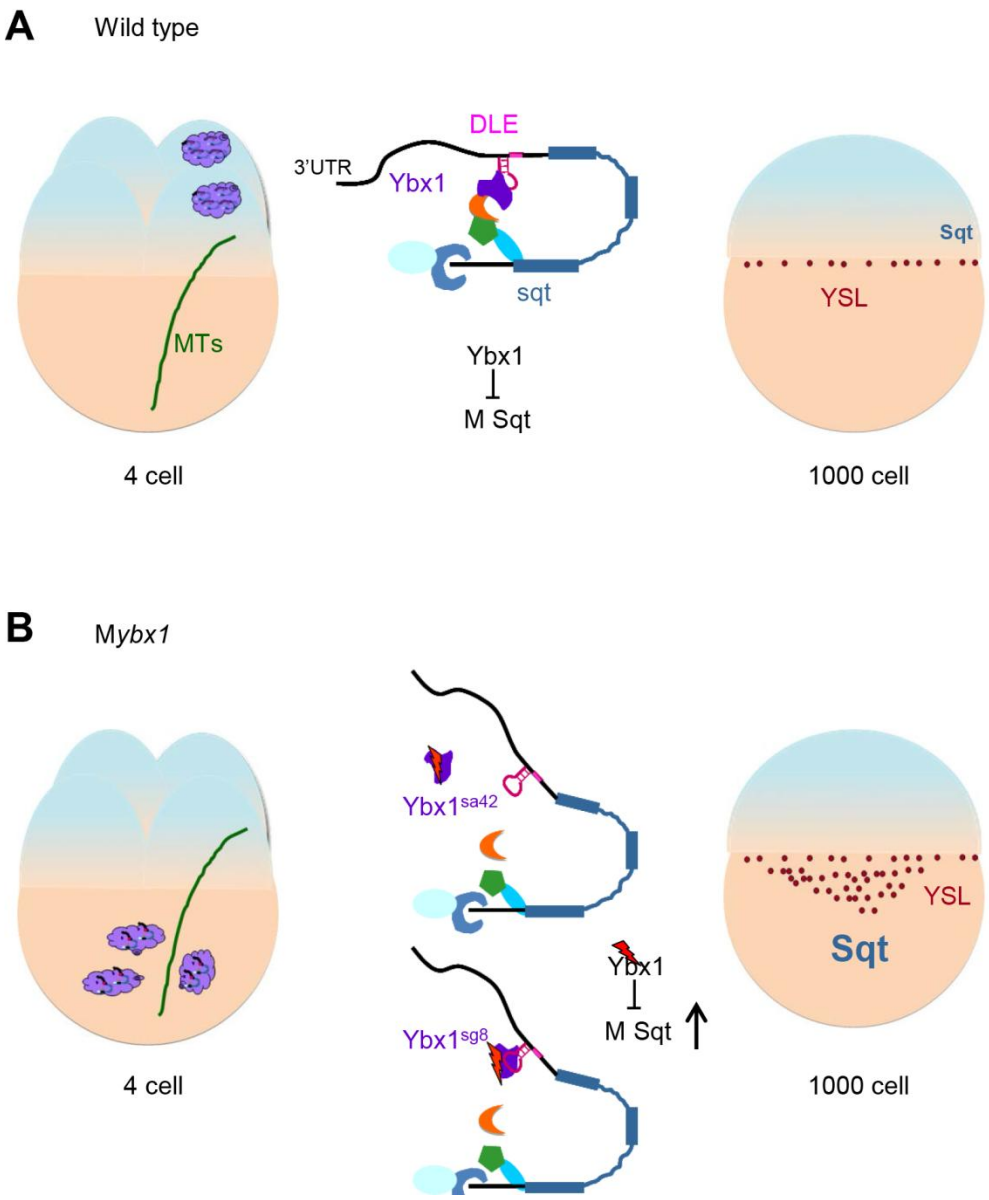

Figure 4.1 – *Graphical Summary*

(A) In wild type embryos, sqt RNA localizes to 2 cells at the 4-cell stage via microtubule cytoskeleton (MTs). DLE lies in the 3'UTR of sqt RNA. Ybx1 binds to sqt-DLE and mediates the formation of sqt RNP granule, wherein maternal sqt RNA is translationally repressed. The embryo develops normally and a single tier of YSL is observed at 1000-cell stage. (B) In M*ybx1* mutant embryos, sqt RNA is not localized and forms aggregates in yolk. The sqt RNP granule fails to assemble and sqt RNA is precociously translated leading to deregulated Nodal signaling and consequent defects in YSL and gastrulation.

4.8 Evolutionary conserved role of Ybx1 binding to sqt 3'UTR

Human NODAL 3'UTR when fused to heterologous lacZ RNA exhibits dorsal localization in zebrafish embryos similar to the sqt 3'UTR (Gore et al., 2005). This was surprising since NODAL RNA is not localized in early mouse embryos ((Robertson et al., 2003), Cheong and Sampath, unpublished observations) and mammalian embryos are thought to undergo regulative development as cells in the early embryos have the capacity to transfate (Ciemerych et al., 2000; Johnson and McConnell, 2004; Yamanaka et al., 2006). This suggests that factors that bind to sqt DLE can also recognize NODAL 3'UTR. So, I tested nodal 3' UTRs from sea urchin, mouse and human (Figure 4.2) for interaction with specific factors in zebrafish embryo extracts and specifically rYbx1. Interestingly, RNA-gelshifts with WT embryo extracts show an SRBF1-like shift (asterisk in Figure 4.2B) on various nodal probes (designed similar to sqt1 probe) while *ybx1*^{sa42} embryo extracts do not show similar binding activity. To further confirm that the SRBF1 like activity is a result of Ybx1 binding to various nodal probes, I used rYbx1 in RNA-gelshift assays. rYbx1 binds to all nodal probes we tested albeit the binding to mouse nodal is very weak (Figure 4.2B). This weak binding may be due to subtle variation in the binding site in the mouse nodal 3'UTR or lack of some essential binding partner.

Ybx1 binds to the DLE, and regulates both localization and translation of sqt RNA. Hence nodal 3'UTRs from other organisms may also harbor a translational control element. Our findings show sqt translation is actively repressed in blastula stages and deregulated Sqt/Nodal signaling leads to embryonic lethality, suggesting an essential role for maternal control of Nodal Signaling. Regulation of Nodal signaling at the transcriptional level and by inhibitors such as Lefty proteins and miRNAs have been well studied (Luo et al., 2012; Schier, 2009b; Shen, 2007). However, translational control is a novel mechanism of regulation of this pathway.

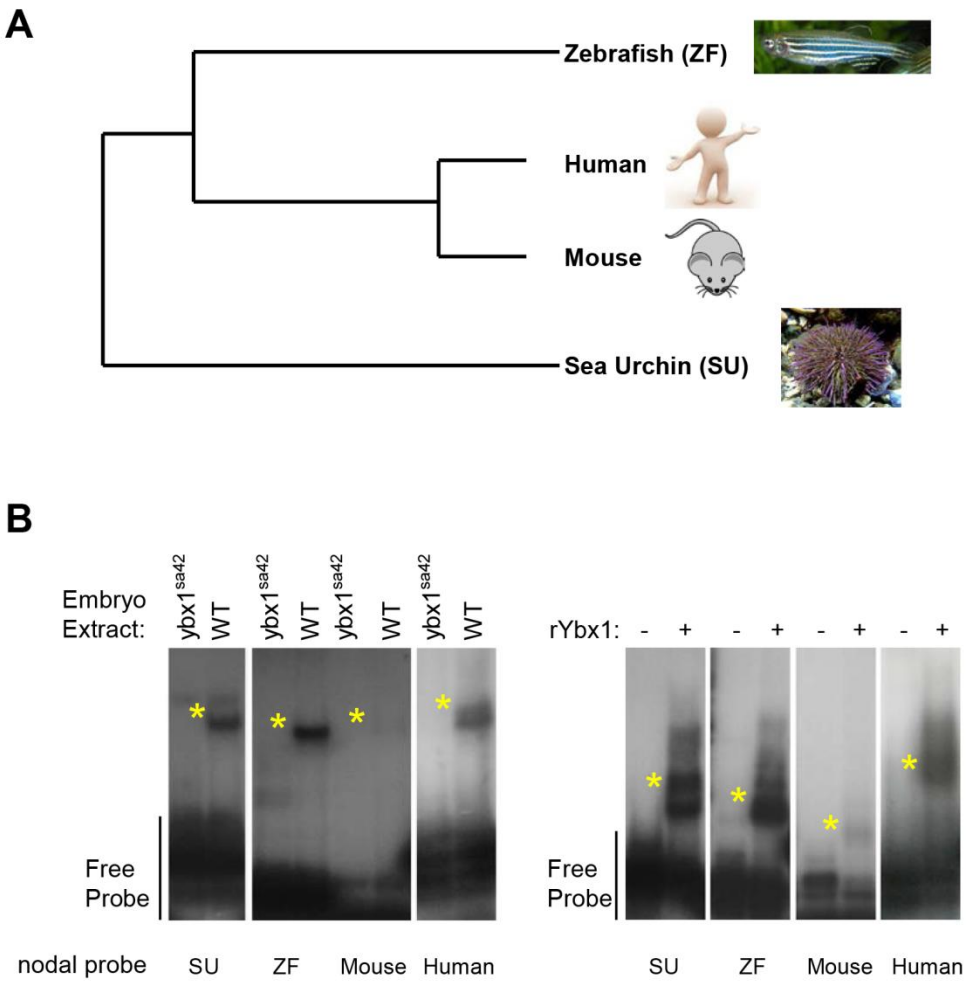

Figure 4.2 – *Ybx1 binding to nodal 3'UTR is conserved*

(A) A schematic showing the phylogenetic relation between *nodal* sequences from sea urchin, zebrafish, mouse and human. (B) RNA gelshift with WT embryo extract show SRBF1 like binding activity (yellow asterisk) on probes from sea urchin, mouse and human nodal 3'UTR but not with *ybx1*^{sa42} embryo extracts. rYbx1 also binds to probes from sea urchin, mouse and human nodal UTRs. Binding to mouse nodal probe is weak. All probes used are similar in design to sqt1 probe in Figure 3.4A.

4.9 Translational control of nodal signaling: implications in disease and stem cell pluripotency

It will be interesting to investigate if Ybx1 complex regulates Nodal signaling in other organisms or biological processes. NODAL and Activin receptor-like kinase7 (ALK7) receptors are expressed in human ovary and placenta, and pre-eclamptic placentas show elevated levels of NODAL and ALK7 (Munir et al., 2004; Nadeem et al., 2011). Therefore, precise regulation of maternal Nodal signaling is likely to be important for human placentation.

Nodal signaling has been implicated in cancer progression. Nodal pathway is activated in many human cancer and elevated expression of Nodal correlates with malignancy of melanoma (Topczewska et al., 2006). Nodal expression is also seen in malignant cells in context of endometrial and prostate cancer and interestingly these cells lack feedback regulation of Nodal signaling due to lack of Lefty expression (Lawrence et al., 2011; Papageorgiou et al., 2009). These disease conditions associated with deregulated Nodal signaling further emphasize the importance of understanding the precise mechanisms behind Nodal signaling regulation.

Nodal signaling has essential roles in maintenance of human stem cell pluripotency (Brandenberger et al., 2004; James et al., 2005). Overexpression of Nodal either by recombinant protein or constitutively expressed transgene prolongs the undifferentiated state of human stem cells (Vallier et al., 2004). Most current methods to maintain embryonic stem cell (ESC) and induced pluripotent stem cells (iPSC) are based on expression of transcription factors with a potential risk of transformation. Our finding that Nodal signaling is maternally regulated by translational repression could provide an alternative method for manipulation of these important therapeutic cells.

Chapter 5

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