Molecular Mechanism of BMP4/Xom Signal Transduction

Thesis Presented

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# Table of Contents

I. Acknowledgements ................................................................. ii)

II. Abstract .................................................................................... iii)

III. List of the figures ................................................................. iv)

IV. Statement of the problem................................................................. vi)

V. Introduction:

A. Axes formation during early vertebrate embryogenesis ............. 1
  A1. Embryogenesis ................................................................. 1
  A2. Axes formation ................................................................. 3
  A3. Signaling pathways involved in axes formation during early embryogenesis. 7
  A4. The canonical Wnt signaling pathway  ................................ 9
  A5. The BMP4 signaling pathway ............................................. 13
  A6. The LEF1/TCF transcriptional factors ......................... 16

B. Xom, a key transcription factor of BMP4 signaling pathway ...... 18
  B1. Xom protein and its role in early embryogenesis .................... 18
  B2. Ubiquitin-mediated proteolysis and its role in early embryogenesis .... 21
  B3. Xom protein is an unstable protein, and its protein level is ......... 23
      regulated by phosphorylation and Ubiquitin-mediated proteolysis.

C. References .................................................................................. 26

VI. Experimental Methods, Results and Discussion: .......................... 40

A. Chapter 1: Xom interacts with and stimulates transcriptional activity of LEF1/TCFs: implications for ventral cell fate determination during vertebrate embryogenesis .................. 40

B. Chapter 2: Regulation of Xom proteolysis by Setine 140/144 phosphorylation during early embryogenesis .......... 66

C. Chapter 3: Hom-1 inhibits tumor growth in both P53 sufficient and deficient cancer cells ........................................ 95

VII. Summary and Conclusion .......................................................... 119

VIII. Appendix (Overall Material and Methods) .................................... 122
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Abstract

Formation of the body axis is one of the earliest and the most important steps of embryogenesis. BMP4 and Wnt are the two most important signaling pathways governing the body axis formation and cell fate determination. Deregulation of these two pathways is implicated in tumor genesis, birth defect, and regeneration diseases.

Xom, a major transcription factor downstream of BMP4, is a very important homeobox protein with dual roles of transcriptional activation and transcription repression. In spite of the critical role of BMP4/Xom signaling in cell fate determination, the molecular mechanism underlying BMP4/Xom signal transduction is not fully understood.

The aim of the thesis is to further understand how BMP4 signaling is transduced through Xom, and how Xom protein level is regulated by ubiquitin mediated proteolysis. I also explored the function of Hom-1, a human Xom homolog.

From this thesis study, using the Xenopus model, I made three important observations. First, I learned that Xom complexes with LEF/TCFs and activates LEF/TCF medicated transcription. I demonstrated that this functional interaction is essential for axis formation and cell fate determination. As LEF/TCFs are important transcriptional factors medicating Canonical Wnt signaling pathway, my data suggest that LEF/TCFs may function as a point of convergence to mediate the combined signaling of Wnt and BMP4 pathways during early embryogenesis. Second, I determined that Xom proteolysis is regulated by phosphorylation of the critical serine residues of the Xom destruction motif. Third, I observed that human homolog of Xom (Hom-1) exerts growth inhibitory effects on the growth of cancer cells.
List of the Figures

I. Figures in introduction section.

1. Embryogenesis process in mammals.................................................................2
2. Axes formation process.......................................................................................4
3. Radial symmetry of the fertilized egg.................................................................5
4. Established caudal/rostral axis at four-cell stage.............................................6
5. Spemann-Mangold organizer (Rostralization center)........................................7
6. The Wnt/beta-catenin canonical pathway.........................................................11
7. Current model for establishment of caudal/rostral axis....................................13
8. BMP signaling pathway......................................................................................15
9. Schematic presentation of LEF/TCF signaling pathway....................................17
10. Domain distribution of Xom protein.................................................................19
11. Schematic presentation of BMP4/Xom signaling pathway..............................20
12. Schematic presentation of the ubiquitin-mediated proteolysis and the SCF
    complex.............................................................................................................23
13. Xom degradation domains...............................................................................24

II. Figures in results and discussion section.

A. Chapter 1:

1. Activation of LEF1/TCF-mediated transcription by Xom...............................57
2. Interaction between Xom and LEF1/TCF factors............................................58
3. Activation of LEF1/TCF-mediated transcription by Xom is not via beta-catenin
    accumulation....................................................................................................59
4. Identification of Xom N-terminal transactivation domain...............................60
5. Deletion of Xom N-terminal motif has catastrophic effects during gastrulation..........................................................61

B. Chapter 2:

1. Expression of endogenous Xom during early embryogenesis.........................85
2. Identify Xom degradation signal and the role of Ser 140/144 phosphorylation in Xom proteolysis.........................................................86
3. Recognition of Xom Ser140/144 by a Ser140/144 phospho-specific antibody.........................................................87
4. Ser144 is phosphorylated during incubation with post-gastrulation-stage embryonic extracts.........................................................88
5. Ser144 phosphorylation is a prerequisite for Ser140 phosphorylation...........89
6. Xom binds to beta-TRCP when it is primed with post-gastrulation-stage embryonic extracts.................................................................90
7. Xom Kinase activity in the embryonic extracts is stage-specific.................91
8. Dorsal expression of Xom-2sa blocks axis formation.................................92

C. Chapter 3:

1. Hom-1 encodes a nuclear protein...............................................................111
2. Hom-1 inhibits the growth of human cancer cells and activates apoptosis……112
3. Hom-1 inhibits cancer cell growth and induces apoptosis in cancer cells that contain no functional p53.........................................................113
4. Over expression of Hom-1 activates caspase-3...........................................114
5. Hom-1 inhibits tumor growth and induces apoptotic cell death ex vivo........115
The Statement of the Problem

Embryogenesis serves as an excellent model system to dissect signaling and transcription events critical for cell fate determination. Given the similarity between embryogenesis and tumorogenesis, molecular mechanisms of cell fate determination of embryogenesis are relevant to tumorogenesis. In this thesis project, I have studied Xom, an important signaling protein in embryogenesis, and related my observations about Xom, both in discussion and experiments, to tumorogenesis. In more detail, I have dissected the molecular mechanism of the integration of ventral BMP4/Xom and Wnt/beta-catenin signaling pathway during early embryogenesis (Chapter 1). I have explored the molecular mechanism of the regulatory mechanism of Xom degradation (Chapter 2). I have translated some results of embryonic studies into potential clinical relevance by identifying Hom-1, human homolog of Xom, and explored its effect on cancer cells (Chapter 3). My thesis advances an understanding of the role of Xom in embryogenesis and helps to develop studies of its role in tumorogenesis.

One of the fundamental questions in early embryogenesis is the molecular mechanism underlying the establishment of dorso-ventral asymmetry from the symmetrical structure of a fertilized egg. Deregulated signal and transcriptional events of early embryogenesis are frequently implicated in the pathological condition such as oncogenesis. Therefore, determination of the molecular mechanism of early embryogenesis carries broad implication in clinical medicine.

The BMP4/Xom pathway and the Wnt/beta-catenin pathway are the two critical cell fate determination pathways of early embryogenesis. It is known that the Wnt/beta-catenin pathway is a prominent dorsalization signaling pathway, while BMP4/Xom...
signaling leads to ventralization during early embryogenesis. How do these two pathways interact with each other is unknown. Xom is an essential ventral cell fate determination factor. Xom and BMP4 form a positive feed-back loop to establish ventral signaling and to counteract the dorsalization effect of Wnt/beta-catenin signaling. How does Xom exert its function is unknown.

The first chapter of my thesis work focuses on the question: How is Xom signaling transduced and what is the molecular mechanism of the integration of BMP4/Xom and Wnt/beta-catenin pathways? LEF1/TCFs are HMG box-containing transcriptional factors mediating canonical Wnt/beta-catenin signaling during early embryogenesis and tumorigenesis, and LEF1/TCF mediated transcription has been used as a read-out for Wnt/beta-catenin signaling. To find out the intracellular molecular interaction between the Wnt/beta-catenin and BMP4/Xom pathway, I explored the effect of Xom on LEF/TCF mediated transcription by using Xenopus as an animal model.

Since Xom is an essential dorsal-ventral cell fate determination factor in early embryogenesis, the regulatory mechanism of its protein level is of much interest. Previous studies showed that Xom is an unstable protein and that it is degraded in a regulated manner at the onset of gastrulation. Mutagenesis studies showed that Xom contains a destruction motif, and Ser 140/144 of its destruction motif is critical for Xom protein stability. How Xom proteolysis is regulated is unknown.

The second chapter of my thesis work tests the hypothesis that the phosphorylation of Ser 140/144 subjects Xom to ubiquitin-mediated proteolysis, and this regulation plays a critical role during ventral dorsal axis formation. Using an anti-Xom phospho-specific antibody, mass spectrometry, and mutagenesis studies, I analyzed the
critical serine phosphorylation sites of Xom destruction motif, and used Xenopus embryos to explore the biological significance of this phosphorylation during early embryogenesis.

Embryonic cells and cancer cells share remarkable similarities both in phenotype and in molecular origins. Both cells are immortal, undifferentiated and invasive. Based on accumulated knowledge derived from embryology and cancer biology, disturbance of signaling pathways involved in early embryogenesis could lead to cancer. For example, Wnt/beta-catenin signaling is minimal in normal adult differentiated cells, while this signaling is high during embryogenesis. Gain of function mutations of Wnt/beta-catenin signaling are frequently detected in a variety of cancers such as colon cancer, hepatocellular cancer and melanoma. Beta-catenin has served as a target of intervention for cancer prevention and treatment. Given the counteracting nature of Wnt/beta-catenin and BMP4/Xom signaling pathways during early embryogenesis, and potentially in tumorgenesis, we hypothesized that the human Xom homolog (Hom-1) may function as a tumor suppression gene. Therefore, I have explored the effect of Hom-1 on cancer cell growth in chapter three.
II. Introduction:


How the highly organized body is formed from a single cell is one of the greatest miracles of mankind. It was believed during the Renaissance that “an angel of God had to enter the womb to make a living body from the embryo” (Said by Physician Maimonides 1190). Even with the rapid development of modern science, the question remains: “How can matter alone construct the organized tissues of the embryo”? Developmental biologists are starting to answer the question piece by piece, step by step, although it is far from complete.

Embryogenesis is a process by which a single cell develops into a characteristic adult configuration. As detailed in Figure 1, embryogenesis starts with the fertilization of an ovum by a sperm and the fertilized egg is called a zygote. The zygote undergoes rapid cell division and a blastocyst is formed when a spherical layer of cells surrounding a yolk-filled cavity with a cluster of inner cell mass is established. The inner cell mass continues its rapid cell division and starts to differentiate (an unspecialized or less specialized cell becomes a specialized body cell) and migrate, and subsequently forms three germ layers: ectoderm, mesoderm, and endoderm. This process is called gastrulation. During gastrulation, cells start major differentiation, and gradually lose their pluripotentiality (the ability to differentiate into all cell types except embryo cells). The three germ layers continue dividing and differentiating, and become the future epidermis, neural tissues, heart, liver, muscle cells, etc. The latter process is called organogenesis. The embryogenesis process, especially the gastrulation process, is highly regulated, and
deregulation of the process will lead to miscarriage or birth defects (Developmental Biology, Six Edition, by Scott F. Gilbert).

Figure 1: Embryogenesis process in mammals. The fertilized egg (zygote) will develop to blastocyst with the inner mass (the light blue mass). The inner mass will develop into three germ layers (ectoderm, mesoderm, and endoderm). The three germ layers will continue to develop into the specific organ tissues. (the figure is adapted from http://www.ncbi.nlm.nih.gov/About/primer/genetics_cell.html)
A2. Axis formation

All organisms are three-dimensional consisting of three axes: anterior-posterior (caudal-rostral, or head-tail), dorsal-ventral (backbone-belly), and left-right. The three axes describe the body plan all through the development (Lane and Sheets, 2002; Schier and Talbot, 2005).

However, the embryonic egg is a symmetric structure (geometric invariance). One of the earliest and most critical steps occurring during early embryogenesis is the formation of embryonic axes. All critical developmental milestones (formation of three germ layers and specification of organ progenitors) are based on the establishment of the three body axes.

Amphibian embryos (frog or Xenopus) have served as a model system to study vertebral axis patterning for more than a century. With current technology, the amphibian embryos have three advantages for researchers: the abundance of the embryonic tissues (the fertilized eggs); the convenient observation of the embryonic events, as the eggs are growing outside of the mother’s body; and larger size and closer evolutionary relationship to mammals compared with fish models (De Robertis, 2006). As described in Figure 2, a mature unfertilized frog oocyte (egg) is radially symmetrical with animal and vegetal pole. The germinal vesicles (containing maternal factors) accumulating in the heavily pigmented animal hemisphere while yolk material is concentrated in the lightly pigmented vegetal hemisphere. Sperm entrance initiates a reorganization of the egg cytoplasm by triggering massive cytoskeleton, molecular, and biochemical rearrangement during the first cell cycle. This process is called cortical rotation. Shearing between the cytoplasm and the cortex reduces the pigmentation of one
side of the animal hemisphere, which visually demonstrates that cortical rotation has broken the radial symmetry and established the dorsal-ventral axis (replacing the animal/vegetal axis), then rostral/caudal axis, then right/left axis. The sperm entrance point (SPE) marks the future caudal side, while the opposite side marks the future rostral side (De Robertis and Kuroda, 2004; Nishida, 2005; Schier and Talbot, 2005; Weaver and Kimelman, 2004).

**Figure 2: Axes formation process.** A. A fertilized Xenopus egg is radially symmetrical with a dark-colored animal pole and light-colored vegetal pole. B. The sperm entrance marks the establishment of caudal/rostral and dorsal/ventral asymmetry. The double arrows indicate the rearrangement of the cytoplasm content during cortical rotation. C. The body map of a tadpole, indicating the dorsal/ventral and rostral/caudal axis (Lane and Sheets, 2006).
The establishment of the embryonic axes is demonstrated by classic specification experiments. As illustrated in Figure 3, when an embryo is cut in half, it will self-regulate to generate the missing part and grow into identical near normal offspring. This experiment demonstrates the symmetry of a fertilized egg.

![Figure 3](image)

*Figure 3. Radial symmetry of the fertilized egg. As indicated in the figure, a fertilized egg is cut in two along the animal/vegetal pole. The two half pairs will develop into a pair of nearly normal offspring with normal body patterning (De Robertis, 2006).*

In 1983, Kageura and Yamana split a four-cell frog embryo into two-cell pairs, and continued to culture the two-cell embryo in isolation under the same conditions (Figure 4). The cell pair at the rostral side yielded head-trunk body plans with almost complete dorsal-ventral structures; while the cell pair at the caudal side gave trunk-tail body plan without the head. The experiment demonstrated that as early as the four-cell stage, the rostral/caudal axis has been established. Evidence has shown that the dorsal/ventral axis is established even before the four-cell stage. These axes will lead the body plan throughout life.
Figure 4. Established caudal/rostral axis at four-cell stage. A. A four-cell embryo is cut in two along the animal/vegetal pole, and each pair will yield a defective body plan. C&E. The rostral side yields a tadpole without a tail. B&D. The caudal side yields a tadpole without a head (Lane and Sheets, 2006).

For a developing embryo, all the cells have the same genetic material, yet they are quite different in function and morphology. The key factor that controls the difference is the differential activation or suppression of certain sets of genes. How this differential gene expression pattern is established and how a cell fate is determined are the key questions for developmental biologists.

In 1924, Spemann (1935 Nobel Prize winner for the finding) and Mangold demonstrated that cell-cell induction is the key to understanding cell fate determination. In their experiment (Figure 5).

![Image of Spemann-Mangold organizer](image)

*Figure 5. Spemann-Mangold organizer (rostralization center/doralization center). The dorsal lip of a fertilized embryo is transplanted onto the future caudal side of another intact embryo. The tadpole will develop two rostral axes (two heads). The experiment demonstrates that the implanted cells from the Spemann-Mangold organizer can change the fates of other cells (De Robertis and Kuroda, 2004).*

Spemann and Mangold transplanted a group of cells at a specific location (the dorsal lip location) of the future rostral of the embryo to the future caudal side of another embryo. The transplanted cells induced the neighboring cells (future caudal cells) to change their fates. The neighboring cells adopted differential pathways that are more
rostral with the production of the second head (first head from its own rostral fate cells). The group of cells with the induction property is called the organizer or organizing center, and the specific rostral induction cells are called Spemann-Mangold organizer or rostral organizing center (De Robertis and Kuroda, 2004; Nishida, 2005).

How does a cell signal another cell to differentiate into a specific cell type? The current understanding is that there are two ways for cells to communicate with each other. The first is the paracrine induction; the second is the direct cell-cell interaction. Here, we will focus on the paracrine induction, which is the major cell induction pathway. Inducing cells secrete diffusible proteins (called growth and differentiating factors) into the immediate spaces around the cell producing them. The paracrine factors will bind to specific molecules (receptors) on the cell membrane of the neighboring cells, and elicit cascade changes of intracellular molecules by way of protein complexation, protein modification (phosphorylation, acetylation, etc.), and gene expression regulation. Finally, those changes will lead to differential gene expression, which in turn, determines the specific cell fate. (Developmental Biology, Six Edition, by Scott F. Gilbert).

Transcription is the process of making an RNA copy from a sequence of DNA (a gene). Transcription is the first step in gene expression. Transcriptions are controlled by transcription factors, which are proteins that bind to regulatory regions in the genome and help control gene expression (for example, whether genes are transcribed or not). The establishment of body axes, the patterning process, is governed through cascades of signaling and transcriptional events, which control cell proliferation and differentiation that lead to the establishment of different cell fates. Deciphering the molecular
mechanism underlying patterning formation during early embryogenesis presents a fundamental challenge, essential not only for the comprehension of the mechanism underlying the generation and maintenance of dynamic body structure but also for the prevention and management of pathological conditions such as malformation and malignant transformation, which largely result from deregulated patterning process of normal developmental. (Developmental Biology, Six Edition, by Scott F. Gilbert).

Over the past decades, extensive investigation of the molecular mechanisms of developmental patterning has established the role of four key paracrine factors: the Wnt family, the BMP family, the fibroblast growth factors, and the Hedgehog family. For the purpose of our studies, we will focus on the Wnt and BMP4 signaling pathway and their related transcriptional network involved in cell fate determination during early embryogenesis.

A4. The canonical Wnt signaling pathway

The canonical Wnt signaling pathway is a prominent signal that leads to the establishment of developmental axes. The Wnts are families of secreted glycoprotein paracrines (usually 350-400 amino acids in length), which share sequence homology to the mouse Wnt-1 gene (first named as the Int-1) and the Drosophila wingless (wg) gene(Nusse and Varmus, 1982) (van Ooyen and Nusse, 1984) (Cabrera et al., 1987) (Rijsewijk et al., 1987). Over the past ten years, research not only showed the critical role of the Wnt/beta-catenin pathway in embryogenesis, but also its important role in stem cell regulation and tumor genesis. Aberrant accumulation of Wnt/beta-catenin signaling is found in various tumors such as colorectal cancer, breast cancer, ovary cancer, melanoma, and leukemia. Numerous therapeutic drugs that target blocking of the
Wnt/beta-catenin pathway are in the pre- or clinical trial pipeline (Gavert and Ben-Ze'ev, 2007; Herbst and Kolligs, 2007; Takahashi-Yanaga and Sasaguri, 2007).

Wnt signals can activate three independent intracellular pathways. Here, we will focus on only the Canonical Wnt signaling pathway, which is the major Wnt pathway. As shown in Figure 6, the Wnt signal is transduced through the cell membrane receptor, Frizzled (Fz), which encodes a seven-transmembrane protein and is required for asymmetric cell division (Sawa et al., 1996) (Bhanot et al., 1996). Other co-receptors, such as cell surface heparin-sulfate and LRP, are also needed for the transduction of Wnt signaling (Schlessinger et al., 1995) (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The LRP gene encodes a single transmembrane protein that interacts with the Wnt molecule through its extracellular domain (Tamai et al., 2000) (Mao et al., 2001b). In the absence of Wnt signaling, there is a default formation of an intracellular kinase complex consisting of three proteins APC, Axin, and Gak3B. This kinase complex will phosphorylate a protein called beta-catenin, which in turn will be rapidly degraded by ubiquitin mediated proteolysis (a intracellular protein degradation machinery. In contrast, when Wnt protein is secreted, it will bind to its receptor Frizzled and its co-receptor LRP. The complexation will lead to the phosphorylation of Dishevelled (Dsh). The activated Dsh protein will bind to and inhibit the Gsk3b kinase complex, preventing the phosphorylation of beta-catenin (Mao et al., 2001a). Therefore, beta-catenin, now stabilized, will move into the nucleus, complex with TCF transcription factors, and modulate the transcription of Wnt signaling downstream genes such as cyclin D1 (a cell cycle gene), and c-myc (an oncogene), etc. (Amit et al., 2002; Behrens et al., 1998; Kishida et al., 1999; Klingensmith et al., 1994; Pizzuti et al., 1996; Rubinfeld et al., 1996;
Rubinfeld et al., 1993; Willert et al., 1997; Yost et al., 1998) (Liu et al., 2002; Yost et al., 1996).

**Figure 6. The Wnt/b-catenin canonical pathway.** A. Without the presence of the Wnt signaling, beta-catenin is constitutively phosphorylated by the GSK3 kinase complex. The phosphorylation renders beta-catenin to protein degradation machinery. Therefore, there is no cytoplasm accumulation of beta-catenin. B. With the presence of the Wnt signaling, Wnt protein will complex with its receptors Frizzled and LRP5/6, which, in turn, will activate Dsh. Activated Dsh will inhibit the GSK3 kinase complex. This will lead to the accumulation of unphosphorylated beta-catenin, which is resistant to proteolysis. Beta-catenin will get into the nucleus, complex with TCF/Lef proteins, bind to the promoter region of the target genes, and activate a cascade of gene transcription events (Croce and McClay, 2006).
The role and mechanism of Wnt molecules in caudal/rostral patterning have elicited extensive research and debate (Tao et al., 2005) (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991). Injection of the mRNAs of a variety of Wnt genes into the ventral blastomeres leads to axis duplication (Christian and Moon, 1993). Moreover, expression of dominant negative Wnt or secreted Wnt antagonist blocks Wnt-induced axis duplications (Hoppler et al., 1996; Leyns et al., 1997).

Current research suggests a leading role for beta-catenin in the formation of the rostral organization center (Spemann-Mangold organizer). As shown in Figure 7, Miller and Moon et al. described the establishment of the caudal organization center as the consequence of sperm entry-induced cortical rotation (Miller et al., 1999). After the sperm enters the egg, the dynamics will cause the rearrangement of the cytoplasm material, including the relocation of Dsh protein from the vegetal pole area to the future rostral side of the embryos. The local concentration of Dsh, an inhibitor of the glycogen kinase-3 (GSK-3), leads to local high concentration of beta-catenin to initiate the cascade of signaling events of establishment of rostral organization center and rostral cell fate determination. Among other functions, the rostral organizing center secretes antagonists to inhibit caudalization (De Robertis and Kuroda, 2004) (Croce and McClay, 2006; Marikawa, 2006).
Figure 7. Current model for establishment of caudal/rostral axis. At the stage of the oocyte, there is an accumulation of Dsh protein, an inhibitor of beta-catenin kinase complex at the vegetal pole. When a sperm enters, it will cause the rearrangement of cytoplasm content. The Dsh protein will relocate to the future rostral side of the egg. The relocation of Dsh will lead to inhibition of beta-catenin phosphorylation, and therefore a local accumulation of beta-catenin. Local high concentration of beta-catenin will initiate the cascade of signaling events that establish the rostral organization center and determine the fate of the rostral cell (Marikawa, 2006).

A5. The BMP4/Xom signaling pathway

In comparison with the extensively studied signaling events of dorsalization and caudalization, the evidence for the existence of rostral organizing, which is $180^\circ$ opposite to the caudal organizing center (the Spemann-Mangold organizer), has just begun to emerge during the past decade. Because of the lack of transplantation induction activity,
no caudalization organizer was identified previously. Only recently, with the development of knockdown technology, has evidence of the existence of caudal organizer accumulated. The makeup of the caudal organizer was further re-enforced by defining its molecular constituents (Niehrs and Pollet, 1999). Many of the genes expressed in the caudal organization center are members of the BMP signaling genes, such as the homeobox genes Vent/Vox/Vega. (Joly et al., 1993) (Gawantka et al., 1995) (Onichtchouk et al., 1996). Altered expression of these caudal organizer genes leads to disrupted axis patterning. (Collavin and Kirschner, 2003)

As shown in Figure 8, BMP4 is a member of the TGF-β super paracrine family, which includes TGF-β isoforms, activins, and BMPs. These secreted BMP signaling molecules bind to type II serine/threonine kinase receptors on the cell surface, followed by aggregation and activation of type I serine/threonine kinase receptor. This complexation of type I and II receptors leads to activation of R-Smad molecules. The activated R-Smad complexes with Co-Smad and enter the nucleus. The Smad complex can regulate the transcription of downstream genes directly, or it can activate some transcription factors, such as the ventral organizer genes Vent 2/Xom (Waite and Eng, 2003) (Henningfeld et al., 2002) (Wagner, 2007; Wordinger and Clark, 2007; Xiao et al., 2007).
Figure 8. BMP signaling pathway. The BMP molecule will bind to its receptor on the cell membrane, and lead to phosphorylation of BMP receptor I (BMPR I). This phosphorylation will lead to phosphorylation of R-Smad. The phosphorylated R-Smad will complex with Co-Smad, move into the nucleus, and activate a cascade of transcription events. Smurf I and I-Smad are the inhibitors of the Smad complexation process (Xiao et al., 2007).

BMP4 has been identified as a strong ventralizing and caudaling factor (Hogan, 1996). Over-expression of BMP4 at the rostral side of embryos during early embryogenesis leads to suppression of rostralization and to embryos that lack the rostral structure, such as the head and CNS systems (Dale et al., 1992) (Jones et al., 1992). Conversely, expression of mutated BMP-4 receptor leads to loss of caudal tissues and
expansion of rostral structures (Maeno et al., 1994) (Graff et al., 1994). In recent years, the BMP pathway has been the focus of extensive studies as accumulating evidence shows its critical involvement in stem cell proliferation and differentiation, tumor genesis, and organogenesis.

A6. The LEF1/TCF transcription factors.

The LEF1/TCFs are a family of high mobility group (HMG) transcription factors. At least four members of the LEF1/TCF family have been identified in vertebrates (LEF1, TCF1, TCF3, and TCF4). To date, no significant functional difference is found between these four LEF1/TCFs, except for protein sequence and distribution differences. Therefore, we call them LEF1/TCFs. They recognize a specific DNA sequence and bind to it. Previous studies have defined the LEF1/TCFs as architectural transcription factors that may induce sharp bending in the DNA helix, which in turn facilitates the binding of the other transcription factors to the promoter region of certain genes (Behrens et al., 1996; Radtke and Clevers, 2005; van Noort and Clevers, 2002). The LEF1/TCF transcription factors possess little intrinsic transcriptional activities, rather, the LEF1/TCF-mediated transcriptional activities are tightly controlled by their associated factors (Hurlstone and Clevers, 2002). Multiple transcription factors were found to modulate the LEF1/TCF signaling (Kramps et al., 2002; Nishita et al., 2000; Takemaru and Moon, 2000). As shown in Figure 9, currently, two LEF/TCF co-repressors and one activator were identified. In a non-induction state, the LEF1/TCFs are associated with transcriptional repressors, such as the Groucho and CtBP, which maintain the LEF1/TCF-mediated transcription in a repressed state (Brannon et al., 1999; Brantjes et al., 2001; Cavallo et al., 1998; Roose et al., 1998; Waltzer and Bienz, 1998). β-catenin is a well-
defined co-activator of the LEF1/TCF-mediated transcription. β-catenin complexes with LEF1/TCF and activates specific gene expression, such as cyclin D1 and C-MYC. During early embryogenesis, local enrichment of β-catenin in the future rostral side of the embryos allows β-catenin to interact with LEF1/TCFs and to induce the expression of caudal-specific genes, such as Siamois, Twin, and Xnr (Brannon et al., 1997; Harland and Gerhart, 1997; Laurent et al., 1997; McKendry et al., 1997).

Figure 9. Schematic presentation of LEF/TCF signaling pathway. Grouch and CtBP are the two repressors that normally bind to LEF/TCF. The complex binds to the LEF/TCF site of the promoter region of their target genes in chromosome, repressing the transcription of the downstream genes. When Beta-catenin complexes with LEF/TCF, the complex triggers the transcription of the downstream genes.

Although well described for its role in rostralization, the role of LEF1/TCFs in ventral/caudal cell fate determination is less clear. Besides direct activation by the beta-catenin, the LEF1/TCF factors were also found to form a complex with Smad molecules of the BMP pathway (Hussein et al., 2003; Labbe et al., 2000; Nishita et al., 2000). LEF1 shows an expression pattern similar to that of bmp4. Transcription of LEF1 starts right before gastrulation and is enriched at the future ventral-caudal side of the animal.
Genetic data indicate that LEF1 is essential for ventral-caudal patterning (Roel et al., 2002). Several studies indicate potential involvement of LEF1/TCF factors in caudalization. For example, expression profiling showed that members of the LEF1/TCF family are broadly distributed in ventral-caudal regions (Molenaar et al., 1998; Oosterwegel et al., 1993). Mutagenesis studies revealed that LEF1-/-TCF1-/- mice carry caudal defects with neural expansion (Galceran et al., 1999). Loss of function of LEF1 leads to caudal/ventral rather than rostral/dorsal defects in *Xenopus* (Roel et al., 2002). Consistent with the possible involvement of LEF1/TCFs in ventral/caudal cell fate determination, promoter analysis revealed that many ventral/caudal genes, such as Xom and Bambi, contain LEF1/TCF binding sites (Karaulanov et al., 2004). Mutations of the LEF1/TCF binding site of these ventral/caudal genes cause significant inhibition of their responsiveness to the BMP4 signaling (Karaulanov et al., 2004) (Arce et al., 2006; Barolo, 2006; Geng et al., 2003).

**B Xom, a key transcription factor of BMP4 signaling pathway.**

**B1.** Xom protein and its role in early embryogenesis

Xom, also called Ventg-2, Vox, and Xbra-1, is a gene encoding 350 amino acid homeobox protein (a family of proteins containing the homeo domain). A homeo domain, a highly conserved DNA sequence about 180 base pairs in length, encodes a polypeptide that can bind to DNA. Almost all of the homeobox containing proteins are transcription factors involved in developmental regulation and body plan formation (Friedle et al., 1998) (Ladher et al., 1996) (Zhu and Kirschner, 2002) (Schmidt et al., 1995).
Four different groups cloned Xom in 1996. As shown in Figure 10, Xom contains three domains: a homeo domain, a proline-rich domain, and a serine-/theonine-rich domain.

![Domain distribution of Xom protein](image)

Experiments showed that around the gastrulation stage, Xom expression concentrates at the ventral-caudal portion of the embryos in both ectoderm and endomesoderm but is excluded from the Spemann’s organizer (Ladher et al., 1996). The expression pattern of Xom during gastrulation is similar to that of BMP4 (Schmidt et al., 1995). Using micro-injection into the animal pole region of the embryos, it was found that the expression of Xom is stimulated by BMP and Wnt (ventral signaling molecules, immediate early target of BMP4) and is inhibited by the Noggin protein of the dorsal organizer (Ladher et al., 1996). In turn, over-expression of Xom was found to caudalize the animal (block head formation), inhibit the expression of rostral organizer genes such as the Xnot and Chordin, and induce the expression of rostral organizer genes such as the BMP4 (Schmidt et al., 1996). As shown in Figure 11, the promoter region of rostral genes such as the goosecoid contains sequences that respond specifically to the inhibitory effect of BMP4, which is called the BMP responsive inhibitory element (BIE) (Kaufmann et al., 1996). In the presence of BMP signaling, Xom binds to the BIE of dorsal genes and inhibits their expression (Friedle et al., 1998). Over-expression of Xom
results in constitutive inhibition of dorsalization similar to the effect of over-expression of BMP4, and therefore results in embryos lacking dorsal structures such as the head (Dale et al., 1992) (Ladher et al., 1996) (Zhu and Kirschner, 2002).

**Figure 11. Schematic presentation of BMP4/XOM signaling pathway.** The Bmp4 molecule binds to its receptor on the cell membrane. Through multiple signal transduction steps, this complexation leads to activation of Xom. Xom binds to BIE (BMP responsive inhibitory element) of caudal-specific genes and inhibits their expression. Meanwhile, Xom also activates the expression of rostral-specific genes such as Vent-1 and BMP4. The specific mechanism is unknown.

As a central player of the caudal organizing center, Xom suppresses the expression of rostral specific genes and activates the expression of caudal genes such as Vent-1, bmp4, and Msx2 (Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996). The molecular mechanism of the transcriptional activation of caudal-ventral specific genes is not well understood.
B2. Ubiquitin-mediated proteolysis and its role in early embryogenesis

In the past 20 years, it has been known that degradation of intracellular proteins is a highly complex, temporally controlled, and tightly regulated process that plays major roles in a broad array of basic pathways during cell life and death. Ubiquitin-mediated proteolysis (UMP) is one of the most important protein degradation processes by which ubiquitin polypeptide chains attach to a protein, and thereby target it for degradation by the 26S proteosome (Hershko and Ciechanover, 1998; Hochstrasser, 1996; Peters et al., 1998). The critical role of UMP in cell fate determination during early embryogenesis is indicated by its role in controlling the protein levels of critical cell fate determination factors such as the beta-catenin of the dorsal organizing center (Behrens, 2000). Besides its role in early embryogenesis, deregulated proteolysis of embryonic cell fate determination factors (such as the β-catenin) has also been implicated in malignant transformation of a variety of cancers such as colorectal cancer, melanoma, and hepatocellular carcinoma. (Bienz and Clevers, 2000; Kinzler and Vogelstein, 1996; Morin, 1999). Clinically, trials of proteosome inhibitors have shown promising results on a variety of solitary cancers as well as chemo resistant hematological malignancies (Cusack, 2003; Dalton, 2003; Nalepa and Harper, 2003; Pagano and Benmaamar, 2003) (Glickman and Ciechanover, 2002).

Regulated protein degradation by the ubiquitin-mediated pathway requires the concerted actions of at least three classes of enzymes: E1, ubiquitin-activating enzyme (which activates ubiquitin in an ATP-dependent manner to generate a high-energy thiol ester); E2, ubiquitin transferase (which transfers activated ubiquitin to the substrate); and E3, ubiquitin-protein ligase (which binds to the target protein specifically, and renders the
target protein to covalent conjugation by ubiquitins). These enzymes transfer polyubiquitin to the lysine residues of the targeting protein and lead to their degradation by the 26S proteasome (proteolysis complex) (Scheffner et al., 1995). Of these three enzymes, E3 probably has the most regulatory importance (Harper and Elledge, 1999). There are at least three major classes of E3s: the HECT domain proteins, the anaphase promoting complex, and the SCF (SKP1-CUL1-F-box protein) complex (Harper and Elledge, 1999). Here, we review the mechanism of the SCF E3 complex, as Xom is degraded by this complex.

As shown in Figure 12, SCF E3 complexes contain three essential components: SKP1, CUL1, and an F-box protein such as the β-TRCP (Bai et al., 1996; Feldman et al., 1997). Degradation of SCF substrates is often regulated by the phosphorylation of the substrates. For example, among SCF-β-TRCP substrates are the critical cell fate determination factors such as the β-catenin, IκBα (Laney and Hochstrasser, 1999), Emi1 (Margottin-Goguet et al., 2003), as well as Xom, which was identified as an UMP-controlled protein through a biochemical screen (Zhu and Kirschner, 2002). Degradation of all the SCF substrates is frequently regulated by phosphorylation of the critical serine/threonine residues of their destruction motifs.
Figure 12. Schematic presentation of the ubiquitin-mediated proteolysis and the SCF complex. UMP requires the concerted actions of at least three classes of enzymes: E1, ubiquitin-activating enzyme; E2, ubiquitin transferase; and E3, ubiquitin ligase. SCF complex is a class of E3 enzyme. SCF complex consists of three proteins: SKP1, CUL1, and an F-box protein. Through E1, E2 and E3, ubiquitin polypeptide will be covalently conjugated to a specific target protein (substrate). The proteolysis machinery will recognize the ubiquitin, and degrade the target protein specifically.

B3. Xom protein is unstable, and its protein level is regulated by phosphorylation and ubiquitin-mediated proteolysis.

Recently, it was found that Xom is an unstable protein and its protein level is also regulated by UMP (Zhu and Kirschner, 2002).

As shown in Figure 13 A, Xom contains two PEST domains (sequences enriched in amino acids proline, glutamic acid, serine, and threonine, which act as proteolytic recognition domain within the polypeptide. These domains are frequently present in unstable proteins. The first PEST domain is located at the amino-terminal of the protein.
(amino acids 29-86), and the second is located in the middle of the molecule (amino acids 131-172) and is critical for Xom stability (Zhu and Kirschner, 2002). When amino acids 132 to 158 were deleted from Xom, the mutant became resistant to ubiquitin-mediated proteolysis. Thus, sequence 132 to 158 was recognized as the Xom destruction motif (Zhu and Kirschner, 2002).

**Figure 13** Xom degradation domains. A. Schematic domain presentation of Xom. Xom is an unstable protein with two PEST domains. Current evidence indicates that PEST2 (amino acid 131-174) is the Xom destruction domain. B. Amino acid sequence comparison of the destruction motif between Xom and β-catenin. Destruction motif is the amino acid sequence recognized by the proteolytic machinery targeting for degradation. In these two proteins, the PEST domain is the destruction motif.

As shown in Figure 13 B, the Xom destruction motif shares strong homology with the destruction motif of β-catenin. Previous studies showed that β-catenin degradation is mediated by the SCF-β-TRCP complex in a Serine 33 and Serine 37 phosphorylation-dependent manner (Winston et al., 1999) (Liu et al., 1999). Similar to the degradation of β-catenin, previous studies showed that when Ser 140/144 of the Xom destruction motif
were changed into alanines, the mutated Xom (named Xom2sa) became resistant to proteolysis, suggesting that, similar to the degradation of beta-catenin, Ser140/144 phosphorylation plays a regulatory role in Xom degradation. This hypothesis was further pursued and proved in our current study (see in chapter II).
References:


Chapter I

Xom interacts with and stimulates transcriptional activity of LEF1/TCFs: implications for ventral cell fate determination during vertebrate embryogenesis
Abstract

LEF1/TCFs are HMG box-containing transcriptional factors mediating canonical Wnt/β-catenin signaling during early embryogenesis and tumorigenesis. β-catenin forms a complex with LEF1/TCFs and transactivates LEF1/TCF-mediated transcriptions during dorsalization. Although LEF/TCF-mediated transcription is also implicated in ventralization, the underlying molecular mechanism is not well understood. Using the vertebrate *Xenopus laevis* model system, we found that Xom, which is a ventralizing homeobox protein with dual role of transcriptional activation and repression, forms a complex with LEF1/TCF through its homeodomain and transactivates LEF1/TCF-mediated transcription through its N-terminal transactivation domain (TAD). Our data show that Xom lacking the N-terminal TAD fails to transactivate ventral genes, such as BMP4 and Xom itself, but retains the ability to suppress transcriptional activation of dorsal gene promoters, such as the Goosecoid promoter, indicating that the Xom transactivation and repression are separable functions. It has been postulated that Xom forms a positive re-enforcement loop with BMP4 to promote ventralization and to suppress dorsal gene expression. Consistent with an essential role of Xom transactivation of LEF1/TCFs during early embryogenesis, we found that expression of the dominant-negative Xom mutant that lacks the LEF1/TCF transactivation domain fails to re-enforce the ventral signaling of BMP4 and causes a catastrophic effect during gastrulation. Our data suggest that the functional interaction of Xom and LEF1/TCF-factors is essential for ventral cell fate determination and that LEF1/TCF factors may function as a point of convergence to mediate the combined signaling of Wnt/β-catenin and BMP4/Xom pathways during early embryogenesis.
Introduction

Xom (also called Vent2, Vox, and Xbr-1), a cell fate determination factor of the Vent family of homeobox genes, is both a transcriptional repressor and an activator [1-4]. During early embryogenesis, Xom is implicated in the formation of ventral mesoderm and in defining the dorsoventral patterning [5, 6]. Zygotic Xom transcription starts after midblastula transition (MBT) and distributes from a more ubiquitous expression pattern during the early gastrula stage to the ventral-lateral regions as gastrulation proceeds [1, 3]. The expression of Xom appears to be positively regulated by signals from the ventral signal center, such as the BMP4, but negatively regulated by dorsal-specific genes, such as the Gooscooid (Gsc) and the noggin [1, 2]. Xom expression in turn contributes to the formation of the dorsoventral pattern by promoting the expression of ventral genes such as the BMP4 and the Vent genes and inhibiting the expression of dorsal-organizer genes such as the Gsc and chordin [2, 3, 7]. To exert its transcriptional repressor function, Xom binds directly to the distal element (DE) of the dorsal specific gene promoters, such as the Gsc, and inhibit their transcription [8]. The molecular mechanism underlying Xom transcriptional activation functions remains to be fully defined.

The LEF1/TCFs are a family of high mobility group (HMG) transcriptional factors that possess no intrinsic transcriptional activities. Rather, the LEF1/TCF-mediated transcription activities are tightly controlled by their associated factors [9]. In a non-induction state, the LEF1/TCFs are associated with transcriptional repressors, such as the Grouch, and CtBP, which maintain the LEF1/TCF-mediated transcription in a repressed state [10-14]. During early embryogenesis, local enrichment of β-catenin in the future dorsal side of embryos allows it to interact with LEF1/TCFs and to induce the expression of dorsal-specific genes, such as Siamois, Twin, and Xnr [15-18]. Besides determination of cell fate during early embryogenesis, excessive activation of LEF1/TCF-mediated transcription by β-catenin has also been implicated as the initial step of malignant transformation of a variety of cancers [19]. The
LEF1/TCF-factors are the transcriptional partners of Wnt/β-catenin, therefore, the LEF1/TCF promoter-luciferase reporter activity has generally been regarded as an indicator of Wnt/β-catenin activities.

The role of LEF1/TCFs in ventral cell fate determination is less clear, although several studies indicate their potential involvement in the process. For example, expression profiling showed that members of LEF1/TCF family are broadly distributed in ventral-posterior regions [20, 21]. Mutagenesis studies revealed that LEF1-/-/TCF1-/- mice carry caudal defects with neural expansion [22] and that loss of function of LEF1 leads to ventral rather than dorsal defects in Xenopus [23]. Consistent with the possible involvement of LEF1/TCFs in ventral cell fate determination, promoter analysis revealed that many ventral genes, such as Xom and Bambi, contain LEF1/TCF binding sites [24]. Mutations of the LEF1/TCF binding site of these ventral genes cause significant inhibition of their responsiveness to the BMP4 signaling [24].

To further define the role of LEF1/TCF factors in ventral cell fate determination, we examined the potential effect of Xom on LEF1/TCF-mediated transcription. We found that Xom forms a complex with LEF1/TCF factors and transactivates LEF1/TCF-mediated transcription. To explore the molecular mechanism of Xom transactivation of LEF1/TCF-mediated transcription, we found that similar to the case of LEF1/TCF transactivation by β-catenin [25], the domains of Xom involved in LEF1/TCF interaction and transactivation are different. We have located the LEF1/TCF-transactivation domain (TAD) of Xom to its N-terminal region, which is separated from the transcriptional repressor domain of Xom. We have further defined that the C-terminal region of LEF1 is involved in interaction with Xom. We found that the Xom TAD is required for transactivation of ventral specific genes, such as the BMP4 and Xom itself. Expression of a dominant-negative Xom that lacks the TAD inhibits BMP4 signaling and disrupts embryogenesis at the gastrulation stage. Our data suggest that functional interaction between Xom and LEF1/TCF-factors plays an essential role in ventral cell fate determination during early embryogenesis. We therefore propose a model in which, the LEF1/TCF transcriptional factors, the previously known transcriptional mediators of the Wnt/beta-catenin signaling, may serve as a point
of convergence to mediate the combined signaling effect of BMP/Xom and Wnt/β-catenin pathways during dorsal-ventral patterning.

Results

**Xom transactivates LEF1/TCF-mediated transcription**

Xom is a major ventral cell fate determination factor of the BMP4 signaling pathway. Using the TOPflash assay, a previous investigation has located LEF1/TCF-mediated transcriptional activities in the ventral-posterior side of embryos [27]. By injecting TOPflash plasmid into the two ventral blastomeres at the 4-cell stage and examining luciferase activities during gastrulation stage, we have also confirmed the LEF1/TCF-mediated transcriptional activities on the ventral side of embryos during early embryogenesis (data not shown, the pGL3-OT, which contains three copies of the optimal TCF binding motif, was a generous gift from Dr. B Vogelstein) [28]. To explore a potential inter-relationship between Xom expression and LEF/TCF-mediated transcription, we examined the effect of Xom expression on LEF1/TCF-mediated transcription using the TOPflash-luciferase assay. When mRNA encoding Xom was co-injected with the TOPflash reporter construct into *Xenopus* embryos at the two-cell stage, expression of Xom enhanced the TOPflash reporter transcriptions sevenfold as compared with its expression in embryos injected with a construct of TOPflash alone (figure 1a). The trans-activating effect of Xom on the TOPflash reporter was specific, since expression of Xom did not activate the control FOPflash reporter (figure 1a) (pGL3-OF, also a gift from Dr. Vogelstein) which contains mutations at the LEF/TCF binding sites) [28]. To determine whether Xom transaction of LEF1/TCF-mediated transcription is context-dependent in embryos, we also studied the effect of Xom expression on LEF1/TCF-mediated transcription in non-embryonic cells. When plasmids encoding Xom and TOPflash were co-transfected into HeLa and HCT116 cells (and later 293T cells) (figure 1b), Xom expression induced the transcription of the TOPflash constructs in these non-embryonic mammalian cells, indicating that Xom exerts a general induction effect on LEF1/TCF-mediated transcription. Again, the specificity of Xom transactivates LEF1/TCF-mediated transcription was indicated by the finding that
Xom failed to activate the FOPflash reporter construct in these tissue culture experiments (data not shown).

**Xom binds to LEF1/TCF factors in vivo and in vitro**

Binding of LEF1/TCFs to the promoter of TOPflash is required for the activation of the reporter construct; thus, the finding that expression of Xom activates TOPflash but not FOPflash raised the possibility that Xom activates the TOPflash reporter through interaction with LEF/TCFs. We tested this hypothesis by determining whether Xom physically interacts with LEF1/TCFs. We carried out co-immunoprecipitation experiments in HCT116 cells transiently transfected with myc-Xom. We found that immunoprecipitation of endogenous TCF4 with anti-TCF4 antibodies co-precipitated the Xom protein (figure 2a-b). The *in vivo* association between Xom and TCF4 appears to be strong and could not be dissociated with 300 mM NaCl and 0.1% NP40. Our previous investigations showed that Xom is an unstable protein and that Xom Ser140/144 to alanine mutation renders the Xom mutant (Xs) resistant to ubiquitin-mediated proteolysis [26]. To determine whether Ser140/144 to alanine mutation interferes with the interaction between Xom and LEF1/TCF-factors, we have also included myc-tagged Xs in the co-immunoprecipitation experiments. Our results showed that myc-Xs binds to TCF4 as efficiently as the wild-type Xom (figure 2b), indicating that the Ser140/144 to alanine mutation does not interfere with the interaction between Xom and LEF1/TCF-factors. To determine the domain of Xom that mediates its interaction with LEF1/TCFs, we made serial deletion mutants of Xom and tested their interactions with TCF4 *in vivo* (figure 2a, c). All Xom mutants that carry the homeobox region co-immuno-precipitated with anti-TCF4 beads, while those that lack the homeodomain did not. These data indicate that the homeodomain of Xom plays a critical role in mediating complex formation between Xom and LEF1/TCF factors. Given that the homeobox domain is a DNA binding domain of Xom, we set to rule out a possible interference of nuclear acids in the interaction between Xom homeodomain and TCF4 by including 20 µg/ml Ethidime Bromide (EtB) in the pull down assays [29]. We found that EtB did not interrupt the interaction between TCF4 and Xom deletion mutants that carry the homeodomain, indicating that the interaction between Xom and TCF4 is not dependent on their potential association.
with DNA (data not shown). Following the demonstration of complex formation between Xom and TCF4 in vivo, we went further to identify the critical domains of LEF1/TCFs involved in the interaction with Xom. At least four members of the LEF1/TCF family have been identified in vertebrates (LEF1, TCF1, TCF3, and TCF4), and LEF1 shows an expression pattern similar to that of Xom. Zygotic transcription of LEF1 starts after the onset of the MBT and is enriched at the ventral-caudal side of the animal [20]. Genetic data indicate that LEF1 is essential for ventral-posterior patterning [23], making LEF1 a potential candidate as the Xom-interacting protein. We therefore tested whether Xom interacts with LEF1 and explored the critical domain of LEF1 involved in the interaction through deletion mutagenesis analysis (figure 2 d, e). During our pilot cotransfection studies, we found that very few cells co-expressed Xom and full-length LEF1 (data not shown); therefore, we probed the potential interaction between Xom and LEF1 through an in vitro binding assay. When in vitro translation (IVT) products of 35S-labeled LEF1 or its deletion mutants were mixed with GST-Xom or GST alone, LEF1 was pulled down by GST-Xom but not by the control GST (figure 2e). The C-terminal–deleted mutant LEF1ΔHMG appeared to have much less affinity for Xom, whereas the N-terminal–deleted mutant of LEF1ΔN60 and LEF1ΔN160 bound to GST-Xom as effectively as did the wild-type LEF1 (figure 2e). Thus, the interaction between Xom and LEF1 appears to be dependent on the LEF1 C-terminal motif, a supposition further supported by a LEF1/TCF transactivation assay (figure 3d). To determine the potential physiological relevance of Xom transactivation of LEF1/TCF-mediated transcription, we examined the effect of Xom on the expression of BMP4 downstream genes, using promoter-luciferase analysis. The Msx2 gene is downstream of BMP4, whose promoter contains both an LEF1/TCF binding site and a BMP4-responsive element (SBE) [30]. Consistent with the potential direct involvement of LEF1/TCFs in BMP/Xom signaling, we found that Xom activates both the wild-type Msx2 promoter and the SBE-mutated Msx2 promoter that retain only functional LEF1/TCF binding sites (data not shown).
Xom transactivates LEF1/TCF-mediated transcription is not mediated through  β-catenin accumulation

Previous studies have shown that LEF1/TCF-mediated transcription is activated by  β-catenin [31-33]. To explore the mechanism of Xom activation of LEF1/TCF-mediated transcription and to rule out the possibility that Xom transactivation of LEF1/TCF-mediated transcription is through an increase in  β-catenin protein level or nuclear translocation, we examined the effect of Xom expression on the protein level and intracellular distribution of  β-catenin in HCT116 cells. As shown in figure 3a-c, whereas Xom expression activates LEF1/TCF-mediated transcription in a concentration-dependent manner (figure 3a), there is no corresponding increase (rather a small decrease) in the level of total intracellular  β-catenin protein (figure 3b). In addition, when the total intracellular proteins were fractionated into the cytoplasmic and nuclear portions, no significant nuclear shifts of  β-catenin were observed upon Xom expression (again, rather a small decrease) (figure 3c). As shown in figure 2, the domain of LEF1 that interacts with Xom locates at the C-terminal region of the LEF1, which is separated from the N-terminal domain involved in interaction with  β-catenin. Consistent with the possibilities that Xom transactivation of LEF1/TCF-mediated transcription through direct interaction, we found that the LEF1NΔ60, a dominant negative LEF1 mutant that blocks  β-catenin transactivation of LEF1/TCF-mediated transcription, promotes Xom transactivation of the LEF1/TCF-mediated transcription in TOPflash assay (figure 3d). In comparison, LEF1ΔHMG mutant reduces Xom transactivation of LEF1/TCF-mediated transcription (figure 3d), most likely through sequestering endogenous LEF1/TCF factors.

Xom transactivates LEF1/TCFs through its N-terminal TAD

The interaction between Xom and LEF1 allowed us to further explored the molecular mechanism of Xom transactivation of LEF1/TCF-mediated transcription. Previous studies have shown that  β-catenin binds to TCFs through its central Armadillo repeats but activates TCFs through its C-terminal motif [25, 34]. Therefore, after identifying the critical domain of Xom involved in LEF1/TCF
interaction, we further analyzed the functional domain of Xom involved in LEF1/TCF transactivation. When cDNA encoding Xom or its deletion mutants was co-transfected with the reporter construct of TOPflash into 293T cells, expression of both Xom and its C-terminal deletion mutant XomCΔ85 activated the TOPflash almost 10-fold more than did the control cells transfected with TOPflash only (figure 5a). In comparison, the ability to activate the LEF1/TCF-mediated transcription was significantly diminished in the N-terminal–deleted mutants of Xom, the XomND55 and XomNΔ175. The XomNΔ175 in particular retains little if any ability to transactivate the LEF1/TCF-mediated transcription (figure 4a). Consistent with a model of Xom transactivates LEF1/TCF-mediated transcription through direct interaction, we found that Xom mutant that lack the LEF1/TCF-interaction motif failed to activate LEF1/TCF-mediated transcription during early embryogenesis (data not shown). Xom is a known transcriptional repressor of dorsal gene expression. To determine whether the dual transcriptional function of Xom is independent of each other, we further examined the effect of these Xom deletion mutants on dorsal gene expression during early embryogenesis. For the in vivo testing, mRNA encoding Xom or its deletion mutants were mixed with mRNA encoding Activin, and then co-injected with Gsc-promoter-luciferase construct into the embryos at the two-cell stage. We found that both the N-terminal–deleted mutants and the C-terminal–deleted mutants of Xom strongly inhibited Activin-induced activation of the Gsc-promoter (figure 4b). These data indicated that the function of Xom transactivation domain is independent of the Xom repressor function, and that the Xom transactivation domain (TAD) resides in its N-terminal region, a notion that was further supported with TOPflash assay during early embryogenesis (figure 4c).

**Xom N-terminal TAD is required for ventral signaling**

Xom is emerging as an essential cell fate determination factor during mesoderm differentiation to promote the expression of ventral specific genes [6]. It has been proposed that Xom forms a positive re-enforcement loop with BMP4 to promote ventral cell fate [3]. To determine whether the Xom N-terminal TAD plays a role in Xom transactivation of ventral specific genes, we examined the effect of
XomND175 on transactivation of the BMP4 promoter during early embryogenesis (the BMP4-luciferase construct was a generous gift of Dr. J. Feng [35, 36]). We found that, in contrast to Xom, XomND175 failed to transactivate the BMP4 promoter (figure 4d). Moreover, in comparison with the luciferase activities in control embryos, embryos injected with XomND175 revealed less luciferase activities, suggesting that expression of XomND175 exerts a dominant-negative effect to block the activity of endogenous Xom on the promotion of BMP4 expression. To further determine the effect of Xom TAD on the expression of downstream genes, each of the two blastomeres of embryos at the two-cell stage were injected with mRNA encoding Xom or XomND175. The embryos were allowed to develop until the gastrula stage (stage 10.5) and total mRNAs were extracted from the injected embryos and non-injected control embryos. The levels of BMP4 and Xom expression were determined by Q-PCR, using Histone-4 as an internal control. Consistent with its role in transactivation of ventral gene expression, expression of wild-type Xom increased the expression of BMP4 (figure 4e). In contrast, expression of XomND175 failed to enhance the expression of BMP4, suggesting that the Xom TAD is required for its transactivation of BMP4 expression. Moreover, consistent with the disruption of the positive auto feedback loop of BMP4 and Xom, expression of XomND175 exerted a strong inhibitory effect on the expression of Xom itself (figure 4f). Together with the notion that LEF1/TCF binding sites on the promoters of ventral specific genes are required for their responsiveness to BMP4 signaling and that loss of function of LEF1 leads to ventral defects [24] [23], our findings suggest that Xom trans-activation of LEF1/TCF-mediated transcription is likely to play a critical role in ventral signaling and ventral cell fate determination.

**Xom transactivation of LEF1/TCFs is essential for gastrulation**

Our biochemical and mutagenesis studies revealed that Xom possesses a LEF1/TCF-transactivation domain in its N-terminal region. We have identified a Xom mutant, XomND175, which possesses negligible LEF1/TCF-transactivation ability but retains transcriptional repressor function. To determine the potential physiological function of Xom transactivation of LEF1/TCF-mediated transcription during early embryogenesis, we used the loss-of-function approach and explored the
effect of XomND175 expression on embryogenesis. We obtained mRNA encoding Xom, XomND175, and other Xom deletion mutants through *in vitro* transcription. The mRNAs were diluted to desired concentration with distal water and injected into one of the two ventral blastomeres at the four-cell stage as indicated (figure 5a). We observed that embryos injected with mRNA of XomND175 but not other Xom mutants showed catastrophic effects during gastrulation (figure 5a). In addition to the mutant specificity phenotype of XomND175, the XomND175 effects appeared to be stage-specific. Unlike XomND175-mRNA, XomND175-cDNA caused little, if any, abnormalities during gastrulation (figure 5a). Closer inspection showed that the embryos injected with XomND175 mRNA progressed through the cleavage and pregastrulation stages; however, as gastrulation begins, many large whitish gray-colored cells appear in the injected side (figure 5b). As gastrulation proceeds, these cells gradually lose a defined cellular appearance, giving the embryos a “rotten” appearance. To define the cellular and histological changes associated with XomND175 expression, we fixed the XomND175-mRNA–injected and control embryos, sliced them into 10-uM thin sections, and stained them with H&E and DAPI. Little histological change was associated with ND175 expression at the cleavage and pregastrulation stages, but many large rounded-up structures were seen at the gastrulation stage, which appeared to be dead cells. None of these large rounded-up structures in ND175-injected embryos stained with DAPI, suggesting that they had lost normal cellular structures, such as the nucleus (figure 5c, arrow head). The embryos appeared to be very sensitive to the effect of XomND175. The 50% effective penetration rate was achieved with less than 50 pg of XomND175 mRNA (data not shown). The finding that expression of a dominant-negative Xom mutant that lacks TAD led to embryonic arrest at the gastrulation stage suggests that Xom transactivation of LEF1/TCF-mediated transcription is essential for gastrulation. Similar findings have been noted for other BMP4 related transcriptional factors, such as Bix3 [37].
Discussion

LEF1/TCFs are essential cell fate determination factors whose transcriptional activities are directed by associated factors. Previous studies have described the role of β-catenin as the transcriptional activator of the LEF1/TCFs and the essential functions of the β-catenin activated LEF1/TCF-mediated transcription in dorsoventral patterning, ontogenesis and malignant transformation. Our current investigation indicates that Xom, a homeobox transcriptional factor with dual role of transcriptional repressor and activator function, activates LEF1/TCF-mediated transcription. We found that Xom forms a complex with LEF1/TCF factors and activates LEF1/TCF-mediated transcription through its N-terminal TAD. Our finding that a dominant-negative Xom mutant that lacks the TAD leads to embryonic development arrest at the gastrulation stage indicates that Xom transactivation of LEF1/TCF-mediated transcription is essential for cell fate determination during early embryogenesis.

LEF1/TCFs are architectural transcriptional factors that may induce sharp bending in the DNA helix [31, 38, 39]. Multiple transcriptional factors were found to modulate the β-catenin/LEF1/TCF signaling [40-42]. The findings that Xom, a transcriptional factors that offsets the β-catenin effects on cell fate, binds to LEF1/TCF factors and transactivates LEF1/TCF-mediated transcription through its N-terminal TAD, therefore, presents a new paradigm to understand LEF1/TCF-transactivation and its consequence in cell fate determination. Distinguished from β-catenin, Xom functions also as a transcriptional repressor. Thus, the finding that Xom transactivates LEF1/TCF-mediated transcription reveals a potential mechanism to account for the dual transcriptional functions of Xom. We have mapped the Xom transactivation domain (TAD) to the N-terminal region of Xom. We have yet to determine whether Xom TAD interacts with the basic transcriptional machinery directly or through other intermediates. Previous investigations have shown that Smads binds to both LEF1/TCFs and Xom/Vent-2 [30, 42-44]. Thus possibilities exist that Smads may modulate Xom transcriptional activation of LEF1/TCF downstream genes, similar to their involvement with the β-catenin transactivation of LEF1/TCFs-mediated transcription [30, 42, 44]. Further investigations of these
questions would help to decipher how multi-signaling pathways may be integrated in cell fate determination.

Xom and BMP4 have been postulated to form a positive re-enforcement loop to promote ventral cell fate and to inhibit dorsal cell fate. Previous studies have shown that Xom and BMP4 promote the expression of each other and enhance the expression of ventral genes, such as the BMP4, Wnt8, Vent1 and Xom itself [2, 3]. The molecular mechanism of Xom transactivation of ventral genes was not clear. Recent mutagenesis analysis of ventral gene promoters revealed the critical role of the LEF1/TCF binding sites of these promoters in their responsiveness to BMP4 signaling [24]. Thus, our findings that Xom binds to LEF1/TCF factors and transactivates LEF1/TCF-mediated transcription suggest a mechanistic explanation of Xom activation of ventral gene expression. The potential involvement of Xom transactivation of LEF1/TCF-mediated transcription in vivo is consistent with the findings of Ramel and Lekven, who showed that depletion of Xom-like molecule (such as Vent and Vox) by morpholino reduces TOP activities in some embryos [45]. The essential role of Xom transactivation of LEF1/TCF-mediated transcription during early embryogenesis was revealed by the catastrophic effect of expression dominant-negative Xom that lacks the LEF1/TCF transactivation domain. The XomND175 therefore may serves as a probe to explore the essential genes and signaling pathways underlying Xom induced cell fate during early embryogenesis.

The molecular mechanism underlying the establishment of dorsoventral asymmetry from the symmetrical fertilized eggs constitutes a fundamental question that is broadly implicated in both the biology of early development as well as pathogenesis of a variety of diseases, such as neogenesis and degeneration. The results of previous investigations revealed the critical role of dorsal and ventral organizers in the establishment of dorsoventral asymmetry. Previous investigations have defined the role of LEF1/TCFs as a component of Wnt/β-catenin signaling pathway during the establishment of dorsal organizing center. Our current investigations present several lines of evidence indicating that LEF1/TCFs participate in also the BMP4/Xom signaling of the ventral signal center (figure 5 d, upper panel). The LEF1/TCFs, a prior known component of the Wnt/β-catenin pathway, therefore, may
function as the point of convergence to integrate the combined signaling effects of the Wnt/β-catenin and BMP4/Xom pathways during the establishment of the dorsal-ventral axis (figure 5 d, lower panel). Both β-catenin and Xom are activators of LEF1/TCF-mediated transcription, therefore, β-catenin and Xom could potentially function in a synergistic or antagonizing fashion in a context dependent manner. The complexity of the BMP/Wnt downstream gene promoters and the availabilities of other putative co-factors may play a critical role in the readout of the combined signaling effects of these two pathways. It has been found that the Wnt and BMP pathways synergize to promote the expression of ventral posterior genes, such as Tbx6, but counteract during the expression of dorsal genes, such as the geminin [46, 47]. Extensive investigations have identified multiple extracellular molecules as the mediator of the cross-talk between the dorsal and ventral signaling, identification of the LEF1/TCF-factors as the convergence point of the dorsal and ventral signaling pathway, therefore, presents a new paradigm to understand the intracellular integration of the dorsal and ventral signaling in cell fate determination during early embryogenesis.
Material and Methods

Preparation of Xenopus embryos, microinjection, and luciferase assays

Protocols for handling embryos and for luciferase assays were essentially as described previously [26]. For a typical cellular luciferase assay, 2 x 10⁵ cells were split into 12-well cell-culture plates and incubated for 24 hours and then transfected with 3 µl of liposome transfection reagent (TransIT1, Mirus), 1 µg of DNA plasmids of selected genes, and 0.3 µg of reporter plasmids (Hela cells require one third while 293T cells require one sixth of the DNA and liposome amount). At 48 hours post-transfection, cells were washed with PBS, lysed with 1x cell lysis buffer (Promega cell lysis buffer), scraped, and collected. After incubation on ice for 30 minutes, cells were cleared by centrifugation at 12,000 g for 15 seconds and transferred to a new tube; then 20 µl of the cell lysate was mixed with 100 µl of Luciferase Assay reagent (Promega), and the luciferase activity was measured by scintillation counting.

Plasmids and recombinant proteins

Xenopus LEF1, LEF1\Delta HMG, LEF1\Delta 60, LEF1\Delta 160, Xom, and its deletion mutants were subcloned by the polymerase chain reaction base technique into the pCS2+ and PGEX4T3 vectors and verified by in vitro translation and sequencing. pGL3-OT and pGL3-OF were generous gifts from Dr. B Vogelstein; p2.4BMP4-Luc was a generous gift from Dr. J Feng; pGL3 promoter MSX2 (WT) and pGL3 promoter MSX2-SDM-600/-766 were generous gifts from Dr. C Sirard.

Preparation of nuclear and cytoplasmic extracts

Cells (4 x 10⁶) were trypsinized and washed twice with PBS and pelleted by centrifugation. Total protein was obtained by lysing the cells in 150 µl of RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH8.0) containing 1x proteinase inhibitors (Roche, proteinase inhibitor cocktail). Cytoplasmic proteins were obtained by incubation of 2 x 10⁶ cells with 150 µl of hypotonic buffer (0.05% NP40, 10 mM HEPES pH7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT) containing 1x proteinase inhibitors on ice for 10 minutes and then centrifuged at 4000 rpm for 2 minutes. The
supernatants were collected and used as cytoplasmic proteins. The nuclear pellets were washed twice with PBS. Nuclear proteins were obtained by incubating the pellet on ice for 60 minutes in 150 µl of RIPA buffer. Levels of β-catenin in fractioned cellular extracts were determined by western blotting using specific antibody (Takara Bio).

**GST pull-down assay**

Five micrograms of GST fusion proteins and 5 µl of 35S-labeled IVT proteins were mixed with 20 µl of 50% glutathione Sepharose 4B beads in 500 µl of binding buffer (50 mM Tris pH 8.0, 100 mM NaCl, 50 mM KCL, 5 mM MgCl2, 1 mM DTT, 10% glycerol and 0.2% NP40). The mixtures were incubated at 4°C for 3 hours and washed with 1x PBS plus 0.2% NP40 four times. Bound material was released with 2x sample buffer, boiled at 95°C for 5 min, centrifuged briefly, and revealed by SDS-PAGE and autoradiography.

**Co-immunoprecipitation**

The affinity-protein G beads were prepared by mixing 20 µl of protein G plus-Agarose beads (Santa Cruz Biotechnology) with 1 µg of corresponding antibody. A total of 2 x 10^6 of cells were lysed with 1x cell lysis buffer (Promega) containing 1x protease inhibitor reagent (Roche), incubated on ice for 30 minutes, sonicated briefly, and centrifuged at 12,000 g for 5 minutes at 4°C. The supernatants were further cleaned with 20 µl of untreated protein G plus-Agarose beads and 2 µg of pre-immune serum for 2 hours at 4°C. The supernatants were then mixed with 20 µl of prelabeled protein G plus-Agarose beads at 4°C overnight. The beads were washed four times with PBS plus 0.2% NP40. Bound proteins were released by 2x sample buffer, boiled at 95°C for 5 minutes centrifuged briefly, and revealed by western-blot analysis with specific antibodies.

**Histological staining**

Embryos were fixed with 3.7% formaldehyde, 0.1 M MOPS (pH 7.4), 2 mM EGTA, and 1 mM MgSO_4 at indicated stages. The embryos were then embedded in paraffin and sectioned at a 10-µm thickness. The sections were stained with hematoxylin and eosin (H&E), and subjected to histological
analysis. The sections were further stained with 2 µg/ml of 4´,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen), to reveal the nucleus of the embryonic cells.

**Analysis of gene expression by real-time PCR**

Total RNA was extracted by Trisol methods. Eight embryos from each treatment group were pooled and homogenized in 1 ml of Trizol (Invitrogen). Chloroform (200 µl) was then added to the sample. After vortex mixing, the samples were centrifuged at 12,000 rpm for 1 minute and the supernatants were collected in new tubes. Isoproponol (500 µl) was added to each sample, mixed, and kept at RT for 10 minutes. Samples were then centrifuged for 30 minutes. The pellets were washed twice with 70% ethanol, air dried, and suspended in 100 µl of ddH2O. The final RNA concentration was determined by measurement at OD_{260}. First-strand cDNA was synthesized with the SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer’s protocol. Briefly, 2.5 µg of total RNA from each sample was used. The final volume of the RT product was 20 µl and was diluted to 200 µl (10 x dilution); 8 µl of the diluted RT product was used for real-time PCR using LightCycler System (Roche) and LightCycler FastStart DNA Master SYBR Green I, according to manufacturer’s instructions. The relative levels of gene expression were calculated by the formula: relative gene expression = 2^{-\Delta C_d} (\Delta C_d= cycle of the specific gene – cycle of the reference Histone-4 gene). Each sample was done in triplicate. The sequences of the primers are available upon request.
Figure 1. Activation of LEF1/TCFmediate transcription by Xom.

(A) Xom activates LEF1/TCF reporter construct (TOPflash) in embryonic cells. TOPflash construct (100 pg) was injected into one of the two blastomeres either alone or in conjunction with 0.4 ng mRNA encoding Xom. Luciferase activity was determined after stage 10, and the luciferase activity in embryos injected with TOPflash alone was designated as one unit. FOPflash plasmid was used as the negative control.

(B) Xom activates LEF1/TCF reporter construct in Hela and HCT116 cells. TOPflash construct was transiently transfected into the indicated cells either alone or in conjunction with plasmids encoding Xom and/or β-catenin as indicated. The luciferase activity was determined 48 hours after transfection. Luciferase activities in cells expressing TOPflash alone were defined as one unit. Error bars indicate standard deviations of representative experiments done in triplicate.
Figure 2. Interaction between Xom and LEF1/TCF factors.

(A) Xom and its deletion mutants used in the study of Xom and LEF1/TCF interaction.

(B,C) Intracellular interaction between Xom and TCF4. HCT116 cells were transfected with plasmids encoding myc-tagged Xom, stable Xom (myc-Xs), or Xom deletion mutants. The endogenous TCF4 was immunoprecipitated with anti-TCF4 beads and washed with indicated buffers; the precipitates were subjected to anti-myc antibody in subsequent western-blot analysis.

(D) Delineation of LEF1 and LEF1 deletion mutants.

(E) In vitro pull-down experiments of GST or GST-Xom with 35S-labeled LEF1 or its deletion mutants; 10% of loading input is indicated.
Figure 3

**TOP Assay in HCT116 Cells**

(A) Xom expression activates LEF1/TCF reporter construct in HCT116 cells in a concentration-dependent manner. 2x10^6 HCT116 cells were transfected with a plasmid encoding Xom. Total cell extracts were harvested with RIPA buffer 48 hours after transfection. The level of β-catenin in each sample was determined by western-blot analysis. β-Actin was used as the loading control.

(B) Effect of Xom expression on total β-catenin protein levels in HCT116 cells. The β-catenin level in each portion was determined. β-Actin was used as the loading control; Histone-1 was used as an indicator of the efficiency of fractionation.

(D) Effects of LEF1 deletion mutants on Xom transactivation of TOPflash construct in 293T cells. Luciferase activities in cells expressing TOPflash alone were defined as one unit. Error bars indicate standard deviations of representative experiments done in triplicate.
Each blastomere of embryos at the two-cell stage was injected with 0.1 ng reporter constructs in the construct. The effects of Xom or its mutants on LEF1/TCFs-mediated transcription were determined after 48 hours, using the TOPflash assay. Luciferase activity in cells expressing TOPflash alone were defined as one unit. Error bars indicate standard deviations of representative experiments done in triplicate. (B) Effect of Xom or its deletion mutants on Activin transactivation of Goosecoid transcription. One blastomere of embryos at the two-cell stage was injected with mRNA encoding Activin (50 pg) and Goosecoid-luciferase reporter construct (100 pg) either alone or in combination with mRNA encoding Xom or its deletion mutants. The effects of Xom or its mutants on LEF1/TCFs-mediated transcription were determined after 48 hours, using the TOPflash assay. Luciferase activity in embryos injected with Goosecoid-luciferase reporter construct and Activin alone was defined as one unit. Error bars indicate standard deviations of representative experiments done in triplicate. (A) 293T cells were cotransfected with TOPFlash reporter construct and plasmids encoding Xom or its deletion mutants. The embryos were cultured to stage 10.5, and total RNA was extracted. The levels of BMP4 (E) and Xom (F) in these embryos were determined by Q-PCR and normalized to the mRNA level of Histone-4. (C, D) Effects of Xom or XomND175 on transcription activation of the TOPflash and BMP4 promoter –luciferase construct. Each blastomere of embryos at the two-cell stage was injected with 0.1 ng reporter constructs in the presence or absence of 0.4 ng of mRNA encoding Xom or XomND175. The embryos were cultured to stage 10.5, and luciferase assays was performed as described, representative data of duplicated experiments were presented. (E, F) Effects of Xom or XomND175 mutant on the expression of endogenous BMP4 and Xom itself. Each blastomere of embryos at the two-cell stage was injected with 0.4 ng of mRNA encoding Xom or XomND175 as indicated. The embryos were cultured to stage 10.5, and total RNA was extracted. The levels of BMP4 (E) and Xom (F) in these embryos were determined by Q-PCR and normalized to the mRNA level of Histone-4.
Figure 5. Deletion of Xom N-terminal motif has catastrophic effects during gastrulation.

(A) Phenotype of gastrula-stage embryos injected with indicated mRNA (0.4 ng) or cDNA (0.1 ng) in one of the two ventral blastomeres at the four-cell stage.

(B) Stage-specific effect of XomND175. One of the two ventral blastomeres at the four-cell stage was injected with mRNA (0.4 ng) encoding XomND175, and the effects were followed.

(C) H & E and DAPI staining of thin section of embryos injected with mRNA encoding Xom or XomND175 as detailed above. Arrows indicate area of loss of cellular structures.

(D) Models of Xom transactivation of LEF1/TCF-mediated transcription (top) and Xom/β-catenin activation of LEF1/TCF-mediated transcription in cell fate determination during early embryogenesis (bottom).
Reference


Chapter II

**Xom Serine 140/144 Phosphorylation regulates Xom stability during Early Embryogenesis**
Abstract

Xom is an essential cell fate determination factor in early embryogenesis. Previous studies showed that Xom is an unstable protein and is degraded in a regulated manner at the onset of gastrulation. Mutagenesis studies showed that Xom contains a destruction motif in which the Ser140/144 is critical for Xom stability. How Xom proteolysis is triggered at the onset of gastrulation is not fully understood. Our current investigations show that Xom contains a degron whose function relies on Ser140/144. Further biochemical analyses indicate that Ser144 and Ser140 are differentially phosphorylated in a stepwise manner. Our data show further that Ser140/144 phosphorylation is required for Xom interaction with β-TRCP, the putative Xom E3 ligase. The pattern of Xom Ser140/144 phosphorylation in staged embryonic extracts correlates with the pattern of Xom degradation, suggesting that phosphorylation of Xom Ser140/144 plays a regulatory role in Xom degradation during early embryogenesis.
Introduction

Ubiquitin-mediated proteolysis (UMP) by the 26S proteosome plays important roles in cell fate determination. Among other important cellular functions, UMP governs cell cycle progression, controls chromosome segregation, mediates DNA damage repair, and regulates signal transduction and transcription (Hershko and Ciechanover, 1998; Hochstrasser, 1996; Peters et al., 1998). The critical role of UMP in cell fate determination during early embryogenesis is indicated by its role in controlling the protein levels of critical cell fate determination factors, such as beta-catenin in the dorsal organizing center (Behrens, 2000). Recently, it was found that Xom, a homeobox protein of the ventral organizing center, is also regulated by UMP (Zhu and Kirschner, 2002). The importance of UMP in early embryogenesis is well-illustrated by the disruption in pattern formation produced by altering the stability of these critical factors (Zhu and Kirschner, 2002).

Regulated protein degradation by the ubiquitin-mediated pathway requires the concerted actions of at least three classes of enzymes: E1, ubiquitin-activating enzyme; E2, ubiquitin transferase; and E3, ubiquitin-protein ligase. These enzymes transfer the polyubiquitin to the lysine residues of substrates and lead to their degradation by the 26S proteasome (Scheffner et al., 1995). Of these three enzymes, E3 is probably of the most regulatory importance (Harper and Elledge, 1999). There are at least three major classes of E3s: the HECT domain proteins (by homology to E6-associated protein carboxyl terminal domain), the anaphase promoting complex, and the SCF (SKP1-CUL1-F-box protein) complex (Harper and Elledge, 1999). SCF E3 complexes contain three essential components: SKP1, CUL1 (or its homologue), and an F-box protein, such as the β-TRCP
Degradation of SCF substrates is often regulated by phosphorylation of the substrates.

The motif of unstable proteins that confers instability is called the degron (Bachmair and Varshavsky, 1989; Suzuki and Varshavsky, 1999). Two elements were found to be essential for the function of the degron: the degradation signal motif (the destruction motif), which is recognized by the E3 ubiquitin-ligase of the degradation machinery; and the ubiquitin acceptor sites (Peters et al., 1998). The sequence composition of the degron appears to be quite variable. Some yeast unstable proteins contain destabilizing N-terminal residues, such as the basic residues (Arg, Lys, His), bulky hydrophobic residues (Phe, Leu, Trp, Tyr), and small uncharged residues (Ala, Ser, Thr), findings that led to the definition of the N-end rule pathway (Gonda et al., 1989; Sheng et al., 2002; Varshavsky et al., 1989). Similarly, anaphase-promoting complex (cyclosome) substrates often contain D-box (RxxLxxxN) and KEN-box (KEN) (Fang et al., 1998; Glotzer et al., 1991; McGarry and Kirschner, 1998; Pfleger and Kirschner, 2000). The SCF substrates often contain a PEST domain, sequences rich in proline, aspartic and glutamic acids, and serine and threonine residues (Rogers et al., 1986). These PEST domains function principally as potential sites of phosphorylation, which triggers the ubiquitination and degradation of these unstable proteins (Hochstrasser and Varshavsky, 1990; Peters et al., 1998). Previous studies showed that Xom contains two PEST domains. The first is located at the amino-terminal end of the protein (amino acids 29-86), and the second is located in the middle of the molecule (amino acids 131-172) and was found to be critical for Xom stability; therefore it was identified as the Xom destruction motif (Zhu and Kirschner, 2002) (Ladher et al., 1996). It was found that the
Ser140/144 of the Xom destruction motif is critical for its stability. Using peptide mimicking, it was also found that SCF-β-TRCP is most likely the cellular E3 that mediates the proteolysis of Xom (Zhu and Kirschner, 2002).

During early embryonic patterning, Xom is a major homeobox transcriptional mediator of BMP4 signaling, a strong ventralizing factor of the TGF-β super-family (Graff, 1997) (Hogan, 1996) (Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996). BMP4 exerts its ventralizing function by binding to cell surface BMP4 receptors, and then through a cascade of signal transduction events, inhibits the expression of dorsal-specific genes such as Xnot and goosecoid (Kaufmann et al., 1996) and XFD (Fainsod et al., 1994) and induces the expression of ventral-specific genes, such as the MyoD and BMP4 itself. In the presence of BMP signaling, Xom binds to the BRE (BMP responsive elements) of the downstream genes to modulate their expression. Recently, we found that Xom, like beta-catenin, is a transcriptional activator of the LEF1/TCF factors, previously known as the dedicated nuclear transcriptional partners of the beta-catenin (Brantjes et al., 2002).

Xom and beta-catenin are the essential constituents of the ventral and dorsal organizing centers, respectively (Koide et al., 2005). Given the opposing role of the dorsal and ventral organizing centers in mesoderm patterning, those findings indicate that UMP is likely to play a critical role in balancing the relative abundance of these two critical dorsal and ventral cell fate determination factors. Therefore, we have further explored the regulatory mechanism underlying Xom proteolysis during early embryogenesis. Our studies showed that the abundance of endogenous Xom protein is controlled by UMP. We obtained biochemical evidence showing that Xom Ser140/144 is
phosphorylated. Our data indicate that Xom Ser140/144 phosphorylation plays a regulatory role in governing Xom stability during early embryogenesis and is essential for the proper establishment of dorsoventral patterning.
Results

Xom protein is transiently detected during early embryogenesis

During previous studies, using in vitro degradation assay and transfection studies, Xom was found to be an unstable protein degraded in a stage-specific manner at the onset of gastrulation. To further determine whether ubiquitin-mediated proteolysis plays a critical role in regulating endogenous Xom levels, we have raised Xom-specific antibodies, using a peptide corresponding to the Xom amino acid sequence 14-28. The Xom antibody was affinity-purified and used to monitor endogenous Xom protein levels in staged embryonic extracts. As shown in figure 1, we found the highest levels of endogenous Xom are present during the pre-gastrulation stage (stage 9). The level of Xom expression declines dramatically with the onset of gastrulation at stage 10. Xom becomes undetectable with Xom-specific antibody after stage 11. Previous investigators and our studies showed that, compared with levels of Xom protein, Xom mRNA levels stay high until after stage 17 then gradually decline (Ladher et al., 1996). Therefore, the finding that Xom protein levels stay high for only a short period suggest that proteolysis plays an important role in controlling the expression and function of Xom.

Xom contains a degron whose function is related to Ser140/144

To determine whether Xom proteolysis occurs in a regulated manner or as a result of a default pathway, we have explored a potential regulatory mechanism underlying Xom proteolysis. Previous investigations have identified the Xom destruction motif (amino acids sequence 131 to 152) and the two serines within the destruction motif (ser140/144) that are critical for Xom stability. These findings led to the hypothesis that phosphorylation of Ser140/144 triggers Xom degradation. To prove this hypothesis, we
made a chimerical molecule of six-myc fused in-frame with 120 amino acids from the Xom sequence (amino acids 115 to 242) that spans the Xom destruction motif. The chimerical molecule was named mycXomDM120. As shown in Figure 2, when mycXom120 was incubated with post-gastrulation-stage embryonic extract (stage 11) for 60 minutes, it became poly-ubiquitinated. Moreover, when all the Ser140/144 of the mycXomDM120 were replaced with alanines (SA mutants), the mutated chimera molecule became resistant to ubiquitination. These results suggest that Xom contains a transferable degradation signal sequence, a degron, whose function depends on Ser140/144 phosphorylation. Consistent with Xom degron confers instability to the chimerical molecule, in vitro degradation assays showed that incubation of the mycXomDM120 (but not the mutated mycXomDM120sa) with stage 11 extracts significant reduces protein levels of the chimerical molecule.

Interestingly, the overall topology of the degron appears to be important in rendering the degradation signal. When the degron sequence was truncated to 60 amino acids (spanning amino acids 115 to 182), polyubiquitinaion of the chimerical molecule, mycXomDM60, which contains wild-type serine 140/144, was significantly reduced during incubation with stage 11 extracts.

Xom Ser140/144 is phosphorylated during incubation with post-gastrulation-stage embryonic extracts

Previous mutagenesis analysis and phospho-peptide binding assays indicate that phosphorylation of Ser140/144 signals Xom ubiquitination and degradation. The importance of Ser140/14 in signaling Xom degradation was further proven by our current findings that Ser140/144-to-alanine mutations abolish the Xom degron function. To
further prove the role of Xom Ser140/144 phosphorylation in Xom degradation, we set out to obtain biochemical evidence of Ser140/144 phosphorylation. To determine whether Ser140/144 is phosphorylated during incubation with embryonic extracts, we raised Ser140/144 phospho-specific antibody with a phospho-peptide that contains phospho-serines at the positions Ser140 and Ser144. The crude anti-sera were further purified by affinity purification, and the purified Ser140/144 phospho-specific antibody was named XPS1140/144. Using XPS1140/144, we found that only GST-mycXomDM120 protein but not the GST-Xom120sa (a Ser140/144-to-alanine mutant) was recognized by the phospho-specific antibody after a brief incubation with embryonic extracts. The specific recognition of the GST-Xom120 by the S XPS1140/144 was significantly reduced by exposing the fusion proteins to alkaline phosphatase for 15 minutes, indicating that Xom Ser140/144 was indeed phosphorylated during the incubation (Figure 3). Whether the Ser140 and Ser144 were both phosphorylated during the incubation in the embryonic extracts was further determined by mass spectrometry analysis.

**Mass spectrometry analysis reveals that Xom Ser144 is phosphorylated during incubation with post-gastrulation stage embryonic extracts.**

For mass spectrometry analysis, the GST-Xom120 fusion proteins were incubated with embryonic extracts and were separated on SDS-PAGE gel. The protein band with the right molecular weight were collected and subjected to trypsin and Asp N digestion. The treatment yielded a 20-mer peptide, which was subjected to FT MS/MS (Fourier-transform ion cyclotron mass spectrometry). As shown in figure 4, compared with the control from trypsin digestion of untreated GST-Xom (A), treatment of Xom with
embryonic extracts yielded a phosphopeptide with an 80-Da mass shift at Ser 144. A product ion-scan yielded a clear y and b ion series carrying a phosphate modification at Ser 144. The results indicate that Xom Ser144 was indeed phosphorylated during induction in the embryonic extracts. However, we were not able to demonstrate Ser140 phosphorylation by mass spectrometry analysis, indicating that Ser140 is either under (or non)-phosphorylated or transiently phosphorylated, which eludes detection by the mass spectrometry analysis.

We have used ³²P labeling in vitro phosphorylation experiments to further resolve the relationship between Ser140 and Ser144 phosphorylation. As shown in Figure 5, we found that while Ser140-to-alanine140 mutation does not affect Ser144 phosphorylation, Ser144-to-alanine144 phosphorylation completely protects the fusion protein from phosphorylation, indicating that Ser144 phosphorylation is a prerequisite for Ser140 phosphorylation if Ser140 phosphorylation occurred. Therefore, Ser140 phosphorylation may occur in a step-wise fashion or it is not phosphorylated but it is an important topological amino acid for Xom stability.

**Ser140/144 phosphorylation depends upon Xom interaction with β-TRCP**

Previous investigations showed that β-TRCP binds to a phospho-peptide corresponding to the Xom destruction motif that contains phospho-serines at position Ser140 and Ser144 but not the control peptide that contains non-phosphorylated serines 140 and 144. Moreover, it was found that β-TRCP facilitates Xom degradation in vivo, while dominant negative β-TRCP that lacks the F-box domain blocks Xom degradation in vivo. These data suggest that Xom Ser140/144 phosphorylation leads to its recognition by the SCF-β-TRCP complex and subsequent degradation. To obtain direct support for
this hypothesis, we asked whether the interaction between Xom and β-TRCP is phosphorylation-dependent. GST-Xom or GST-XomSA mutants were incubated with embryonic extracts in the presence or absence of ATP. GST-Xom fusion proteins were incubated with $^{35}$S-labeled β-TRCP, either directly or after priming with post-gastrulation-stage embryonic extracts. As shown in figure 6 A, β-TRCP was pulled down with GST-Xom, only after it was treated with post-gastrulation-stage embryonic extracts. The specific recognition was diminished when ATP was excluded from the incubation. β-TRCP was not pulled down by GST-Xom2SA. These findings indicate that phosphorylation of Ser140/144 is required for the binding of β-TRCP with Xom.

To determine whether Xom Ser140/144 phosphorylation plays a role in its recognition by β-TRCP in vivo, we injected mRNA encoding myc-Xom or myc-Xom mutants that contain Ser140- and Ser144-to-alanine mutations together with mRNA encoding β-TRCP. The embryos were allowed to develop until stage 10-10.5. Myc-Xom or its mutants were immunoprecipitated with myc-antibody, and β-TRCP in the immunocomplex was detected (or ruled out) with specific antibody. As shown in figure 6 B, we found that β-TRCP was co-immunoprecipitated with myc-Xom only, suggesting that phosphorylation of Xom Ser140/144 plays a critical role in the interaction between Xom and β-TRCP in vivo.

Ser140/144 phosphorylation is regulated during early embryogenesis and is consistent with Xom degradation pattern

Previous investigation showed that Xom degradation accelerates precipitously with the onset of gastrulation. Using an in vitro degradation assay, we found that Xom remained relatively stable during incubation with pre-gastrulation-stage embryonic
extracts (stage 9), but was rapidly degraded during incubation with post-gastrulation-stage embryonic extracts (stage 11). In addition, we found that Xom Ser140/144-to-alanine mutants are resistant to proteolysis. These findings suggest that Xom Ser140/144 phosphorylation plays a regulatory role in Xom stability. To further test this hypothesis, we examined whether Xom Ser140/144 phosphorylation correlates with degradation. As shown in Figure 7, when Xom protein was incubated with staged extracts from stage 7 to stage 11, Xom was hypo-phosphorylated in stage 9 extracts but became readily phosphorylated in stage 11 extracts. The Xom phosphorylation pattern in staged embryonic extracts correlates with Xom degradation pattern in staged embryonic extracts, suggesting that Ser140/144 phosphorylation plays a regulatory role in Xom stability. Although there is Ser140/144 kinase activity in stage 7 extracts (Xom is phosphorylated during incubation with stage 7 extracts), the physiological relevance of this finding remains unknown, since there was no zygotic transcription of Xom protein during this stage.

**Potential for Ser140/144 phosphorylation is required for progress of gastrulation.**

Previous findings that ectopic expression of the stable Xom mutant XomΔ20 at the dorsal side strongly inhibits dorsal development suggest that regulated proteolysis of Xom is essential for dorsoventral patterning. Recently, our investigations showed that Xom also functions as a transcriptional activator. Therefore, to further prove that the XomΔ20 effect is directly related to altered Xom stability rather than blocking potential functional domains, we tested the effect of expression of Xom2sa, the stable Xom mutant that carries single-point mutations, on dorsoventral pattern formation. As shown in figure 8, expression of Xom2SA mutants at the dorsal side of embryos blocks the development
of dorsal structure, suggesting that potential for phosphorylation of Ser140/144, which triggers Xom degradation at gastrulation, is essential for gastrulation.

**Discussion**

Using a biochemical screening method, previous investigations determined that Xom, an essential early vertebrate embryonic cell fate determination factor of the BMP4 signaling pathway, is degraded by ubiquitin-mediated proteolysis (UMP). It was found that expression of degradation-resistant mutants of Xom led to disrupted dorsoventral patterning during early embryogenesis (Zhu and Kirschner, 2002). These findings suggest that, similar to its critical role in governing cell cycle progression, chromosome segregation, DNA damage repair, signal transduction, and transcription (Hershko and Ciechanover, 1998; Hochstrasser, 1996; Peters et al., 1998), UMP plays an essential role in embryogenesis through controlling the abundance of critical cell fate determination factors during early embryogenesis. Our current studies were designed to further define the regulatory mechanism underlying Xom proteolysis.

*Defining the Xom degron.* Previous studies showed that deletion of the Xom destruction motif leads Xom resistant to UMP. However, it was not clear whether the effect of the deletion is related to removal of a Xom degradation signal or destruction of the topology required for Xom degradation. To distinguish between these two possibilities, we asked whether Xom contains a transferable degradation signal sequence (the degron). We have used a chimera approach, making fusion proteins of candidate Xom degron with a 6-myc tag. Our results show that Xom contains a degron capable of transferring instability to other polypeptides. We have further shown that a certain length...
is required for the function of the Xom degron: amino acids 115 to 242. A shorter polypeptide (amino acids 115 to 181) that contains the Xom destruction motif did not confer instability to the chimera proteins. The minimal sequence required for the degradation of Xom remains to be fully defined.

**Characterizing Xom Ser140/144 phosphorylation.** Degradation of proteins containing PEST domains is often regulated by phosphorylation of critical serine/threonine residues of the PEST domain (Rogers et al., 1986) (Hochstrasser and Varshavsky, 1990; Peters et al., 1998). Previous studies showed that Xom contains two PEST domains, the second of which was designated the Xom destruction motif (Zhu and Kirschner, 2002). Serial mutagenesis studies showed that the Ser140/144 of the Xom destruction motif is critical for stability. However, using peptide mimicking, it was found that Ser140/144 phosphorylation is critical for the recognition of Xom by the SCF-β-TRCP, the putative Xom E3 ligase, it was unclear whether Xom Ser1140/144 was phosphorylated or whether the phosphorylation plays a role in Xom ubiquitination and degradation. To answer these questions, we have further characterized the potential phosphorylation of Ser140/144. Using a specific Xom Ser140/144 phosho-antibody, XPS140/144, we found that Xom Ser140/144 was recognized by XPS140/144 after incubation with staged embryonic extracts. The recognition was diminished after exposing the reaction mix to alkaline phosphatase, indicating that Xom Ser140/144 was indeed phosphorylated during the incubation with embryonic extracts. Since XPS140/144 does not distinguish between Ser140 and Ser144 phosphorylation, we further examined the potential phosphorylation of Ser140/144 with mass spectrometry. We have obtained evidence of the addition of a phosphor group to the Ser144 after Xom
incubation in embryonic extracts. Nevertheless, we have been unable to obtain direct evidence of the addition of a phosphor group to the Ser140 after Xom incubation in embryonic extracts. There are two possible explanations for the difference between genetic studies and current biochemical data. First, Ser140 phosphorylation might be a transient process, and the level of phosphorylation may fall below the detection of mass spectrometry analysis. Second, phosphorylation of Ser140 may not be required for the proteolysis of Xom; rather, mutation of Ser140 to alanine altered the topology of the Xom destruction motif and made Xom resistant to UMP. Regardless of which of these two potential mechanisms actually operates, our current data further advanced genetic studies and helped to reveal the difference between Ser140 and Ser144 in mediating Xom proteolysis. Consistently, we found that mutation of Ser140 to alanine does not affect the phosphorylation of Ser144. However, mutation of Ser144 to alanine completely blocks phosphorylation of the Xom destruction motif. These findings suggest that if Xom Ser140 is phosphorylated, it occurs in a stepwise manner after the phosphorylation of Ser144. The complex phosphorylation of the Xom destruction motif suggests that regulation of Xom stability is also a complex process. Consistent with the critical role of Ser140/144 phosphorylation in the degradation of Xom, we have further proven that Ser140/144 phosphorylation is required for Xom recognition by its E3 ligase, β-TRCP.

Ser140/144 phosphorylation is regulated and required for dorsoventral patterning. Xom is an essential constituent of the ventral organizing centers (Koide et al., 2005). Previous studies showed that Xom is degraded at the onset of gastrulation and that its timely destruction is required for the establishment of the dorsoventral axis (Zhu and Kirschner, 2002). However, the mechanism underlying the regulated proteolysis of
Xom remains unclear. The finding that Xom degradation is related to Ser140/144 phosphorylation suggests that Ser140/144 may play a regulatory role in Xom proteolysis. To test this hypothesis, we used the Xom Ser140/144 phospho-specific antibody XPS140/144 to examine the Xom phosphorylation pattern in staged embryonic extracts. We found that Ser140/144 was under-phosphorylated in pre-gastrulation extracts (stage 9) and became phosphorylated after the onset of gastrulation (stage 11). The Xom Ser140/144 phosphorylation pattern correlates with the Xom degradation pattern, suggesting that Ser140/144 phosphorylation plays a regulatory role in Xom degradation. Using an in vitro degradation assay, we found that Xom Ser140/144 is also phosphorylated in extracts prior to mid-blastula transition (MBT) (stage 7), suggesting Xom Ser140/144 kinase activity in stage 7 extracts. Since Xom is not expressed until after the onset of MBT, Xom kinase activity in pre-MBT extracts may relate to physiological functions other than controlling Xom stability. Consistent with Ser140/144 phosphorylation playing a critical role in regulating Xom stability and dorsoventral patterning, we found that expression of a Xom mutant that carries Ser140/144 mutations leads to severe disruption of dorsoventral axis formation. Thus, our current data further extend previous studies and define a regulatory role for Ser140/144 in Xom proteolysis during early embryogenesis.
Material and methods:

Embryonic manipulation

Methods of embryo manipulation and extract preparation were as previously described (zhu).

Raise and purification of Xom antibody and phosphor-specific Xom antibody

To make phospho-specific antibodies that specifically recognize the phosphorylated Xom Ser140/144, we first synthesized phosphopeptides of the Xom destruct motif in which the Ser140 and Ser144 were replaced with phospho-Ser140 and phospho-Ser144 (KSAYSTSTDS\textsuperscript{p}GYES\textsuperscript{p}ETSC). The phospho-peptides were then coupled to keyhole limpet hemocyanin (KLH), mixed with adjuvant, and used to immunize rabbits according to standard protocol (Persson et al., 1998). After we obtained the anti-serum, we further purified the antibodies by affinity purification. The antibodies were first positively selected through binding to an affinity column of phosphopeptides against which the antibodies were raised. The antibodies were then negatively selected by passing them through affinity peptide columns of non-phosphorylated Xom destruction motif. The purified Xom-phospho-specific antibodies were named XPS140/144.

In vitro ubiquitination assay

Proteins were labeled with \textsuperscript{35}S-methionine in a TNT reaction according to manufacture’s instruction (Promega, CAT # L4600). \textsuperscript{35}S-methionine labeled proteins (1 µl) were incubated with 10 µl post-gastrulation-stage embryonic extract (supplemented with 0.2 mg/ml ubiquitin, energy mix, protease inhibitors) at room temperature for 0 to
60 minutes as indicated. After incubation, the proteins were separated on SDS-PAGE gel and revealed by autoradiography.

**FT-MS/MS**

5 µg of indicated GST fusion protein was mixed with 5 µl of embryonic extract, 2 µl 1 mM ATP and 20 mM HEPES (pH7.4) in a total reaction volume of 25 µl. The mixture was incubated at 37ºC for 2 hours, and subjected to SDS-PAGE separation. The band with the right molecular weight was cut out, and soaked in 200 µl 50% ACN/50% 50 mM ammonium bicarbonate solution for 5 minutes. After a brief spin, the sample was changed to the same ACN solution and kept overnight. The pellets were washed with 300 µl ACN solution, and speed vacuumed at 35ºC for 1 hour. Trypsin (~1 µg/band) solution in 50 mM ammonium bicarbonate was added to each sample. The samples were then incubated at 37ºC overnight. The gel pellets are extracted twice with 5% formic acid/50 % Acetonitrile twice, and speed vacuum dried. Liquid chromatography tandem MS (LC-MS/MS) was performed by using a 7-T LTQ FT hybrid mass spectrometer (Thermo Electron). The digested peptides were loaded onto the resolving column with a capillary auto-sampler binary HPLC pumps with an in-line flow splitter. 17 cm of Reverse-phase Magic c18 material (5 µm, 200Å, Michrom Bioresources) was used in the resolving column. Ten LTQ MS.MS spectra were acquired per cycle in a data-dependant fashion from a preceding Fourier transform MS scan (400-1800 m/a at 100,000 resolution with dynamic exclusion).

**In vitro GST-pull-down assay**

5 µg of GST fusion protein and 5 µl of 35S labeled IVT proteins were mixed with 20 µl of 50% Glutathine Sepharose 4B beads in 500 µl of binding buffer (50mM Tris ph8.0, 150mM KCL, 5mM MgCL2, 0.2% NP40). The mixture was incubated at 4ºC for 3 hours, and the beads were washed four times with 1x PBS + 0.2% NP40. The beads were then
mixed with 2 x sample buffer, boiled at 95°C for 5 minute, briefly spun down, and subjected to SDS-PAGE gel electrophoresis. The gels were vacuum dried, and were subjected to autoradiography.

In vitro Xom-Kinase assay

1 µg of indicated GST fusion protein was mixed with 5 µl of Xenopus embryonic extract, 2 µl γ-32P-ATP (Perkin Elmer, Cat #BLU/NEG 502H) and 20 mM HEPES buffer in total volume of 25 µl. The mixture was incubated at 30°C for 30 minutes, and subjected to SDS-PAGE separation. The SDS-PAGE gel was dried and subjected to autoradiography.
Figures and legends to figures

Figure 1

Expression of endogenous Xom during early embryogenesis. Xenopus laevis eggs were fertilized in vitro, and the embryos were allowed to develop in 0.1X MMR as described previously. The embryos were harvested at each developmental stage as indicated, and the embryonic proteins were analyzed by SDS-PAGE. The level of endogenous Xom protein was measured with a Xom-specific antibody in western blot analysis. Note: the Xom protein level peaks around gastrulation (stage 9 and 10) and then declines rapidly. β-tubulin was used as loading control.
Figure 2. Identify Xom degradation signal and the role of Ser140/144 phosphorylation in Xom proteolysis.

Chimeras of MycXomDM60, MycXomDM60sa, MycXomDM120, MycXomDM120sa, Xom, and Xomsa were incubated with post-gastrulation-stage embryonic extracts (supplemented with ubiquitin, energy mix, and protease inhibitors) at room temperature for 0 to 60 minutes as indicated. After incubation, the proteins were segregated on SDS-PAGE and revealed by autoradiography. The higher molecular bands (smear) indicate the formation of polyubiquitin side chains on the chimera. Note that when the serines 140/144 were changed to alanines, polyubiquitination ceased. Also, when the chimera was shortened to 60 amino acids spanning the Xom destruction motif, the efficiency of ubiquitination was significantly reduced.
Figure 3

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<th>GST-Xom120</th>
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<tr>
<td>Stage 11 extract</td>
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<td>Alk phosphatase</td>
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Figure 3. Recognition of Xom Ser140/144 phosphorylation by a Ser140/144 phospho-specific antibody. GST-Xom120 fusion protein or the mutant GST-Xom120sa fusion protein was incubated with stage-11 extracts at room temperature for one hour. The reaction was followed by alkaline phosphatase treatment for 15 minutes as indicated. The reaction mixtures were separated by SDS-PAGE, and the presence or absence of phospho-Ser140/144 was revealed by XPS140/144 in subsequent western blot analysis. Note, exposure of GST-Xom120 to alkaline phosphatase reduces its recognition by XPS140/144.
Figure 4. Ser144 is phosphorylated during incubation with post-gastrulation-stage embryonic extracts. GST-Xom fusion protein was incubated with (or without) post-gastrulation-stage embryonic extracts for 60 minutes at room temperature. After incubation, the GST fusion protein was separated by SDS-PAGE. The fusion protein was excised and subjected to gel digestion with trypsin and Asp-N. The peptides were extracted with 50%ACN/5% formic acid, vacuum-dried, and subjected to LC-MS/MS analysis. A. y and b ion-scan series of untreated peptide (D139 to G156). B. y and b ion-scan series of treated peptide (D139 to G156). Note: mass shift of 80 Da starting at y13 of the y ion series and the b6 of the b ion series, indicating the Ser144 is phosphorylated during Xom incubation with the embryonic extracts.
Figure 5. Ser144 phosphorylation is a prerequisite for Ser140 phosphorylation. GST-Xom, GST-Xom 2SA, GSTXoms144a, and GST-Xoms140a fusion proteins were incubated with post-gastrulation-stage embryonic extracts in the presence of γ-32P-ATP at room temperature for 30 minutes. After incubation, the GST fusion protein was separated by SDS-PAGE. The phosphorylation status of each fusion protein was revealed with autoradiography. Note: both GST-Xom and GST-Xoms140a were phosphorylated, but GST-Xom 2SA and GST-Xoms1144a were not phosphorylated during the incubation. The findings suggest that Ser144 phosphorylation is a prerequisite for Ser140 phosphorylation.
Figure 6

Figure 6. Xom binds to β-TRCP only after it is primed with post-gastrulation-stage embryonic extracts.

A. In vitro binding of Xom and β-TRCP: GST-Xom and GST-Xom2sa, which contains S140/144-to-alanine mutations, were incubated with gastrulation-stage embryonic extracts. The treated and control untreated GST-Xom fusion proteins were then mixed with 35S-labeled IVT products of β-TRCP at 4°C for two hours. The GST-Xom bound proteins were pulled down and detected by autoradiography. Note: Only extract-treated GST-Xom associated with β-TRCP. The requirement of ATP for the reaction is consistent with an energy-requiring step of phosphorylation.

B. In vivo binding of Xom (X) and β-TRCP: mRNA encoding myc-Xom or myc-Xom serine-to-alanine mutants were injected into embryos together with mRNA encoding β-TRCP. The embryos were cultivated to stage 10-10.5, and myc-Xom or myc-Xom mutants were immunoprecipitated with anti-myc antibodies. The presence or absence of β-TRCP in the immunocomplex was detected with specific anti-β-TRCP antibody in a subsequent IP-western experiment. Note: β-TRCP was pulled down only with myc-Xom in vivo, suggesting that Ser140/144 phosphorylation is important for the recognition of Xom by the β-TRCP in vivo.
Figure 7

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<th>Staged extracts</th>
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<td>GST-Xom</td>
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Figure 7. Xom kinase activity in the embryonic extracts is stage-specific. The fusion protein GST-XomDM120 was incubated with embryonic extracts from stage 7 to 11. The phosphorylation of the Xom destruction motif was detected with XPS140/144 antibody as previously described. Xom was phosphorylated only during incubation with stage 7 and 11 extracts. Note: the hypo-phosphorylated state of Xom during incubation with stage 9 extracts correlates with the relatively slower rate of Xom degradation in stage 9 extracts.
Figure 8

Figure 8. Dorsal expression of Xom2sa blocks axis formation. 0.4 ng of mRNA encoding the Xom2sa mutant was injected into the two dorsal blastomeres at the four-cell stage. The embryos were allowed to develop until tailbud stage, and the effects of expression of Xom2sa were assessed. Note: Nearly all embryos had DAI of 0, indicating that axis formation was blocked.
References:


Chapter III

Hom-1 inhibits tumor growth in both p53 sufficient and deficient cancer cells
Abstract: Homeobox transcriptional factors play an essential role in cell fate determination during early embryogenesis and are implicated in tumorigenesis. Recently, using a Xenopus model, we found that Xom, a homeobox transcriptional factor of the BMP4 pathway, interacts with LEF1/TCF transcriptional factors, previously known as the nuclear mediator of the oncogenic Wnt/beta-catenin pathway, Xom antagonizes beta-catenin in cell fate determination during early embryogenesis. Therefore, we have searched for and identified a human Xom homologue, Hom-1. Transient expression assay showed that Hom-1 expression inhibits the growth of cancer cells and that p53 is not required for its growth inhibitory effects. We further showed that over expression of Hom-1 activates caspase-3 and induces apoptosis both in vitro and ex vivo. Our findings indicate that Hom-1 may serve as a novel target for cancer treatment.
**Introduction:** Homeobox transcriptional factors play essential roles in controlling cell proliferation and differentiation during early embryogenesis and ontogenesis. On the basis of their structural similarities and chromosomal distribution, the vertebrate homeobox proteins are grouped into two groups, the clustered (also called Hox genes) and the unclustered homeobox proteins (Nunes et al., 2003). Expression of homeobox genes is frequently deregulated in cancers, for example, homeobox proteins that are expressed in differentiated cells are often downregulated, while those expressed in undifferentiated cells are often upregulated in cancer (Abate-Shen, 2002); for example, cdx2,4 in colorectal cancer, Nkx3.1 in prostate cancer, KLF 2, 4 in colorectal cancer, and Hoxb4 in hematopoietic malignancies (Nunes, 2003 #106).

Xom is a member of the ventral expressed (vent) family homeobox proteins of the non-clustered group. Xom is expressed during early embryogenesis and is a major downstream mediator of the BMP4 signaling pathway of the ventral signaling center (Ladher et al., 1996). Recently, using a *Xenopus* model, we found that Xom interacts with LEF1/TCF transcriptional factors and plays an essential role during early embryogenesis (Gao et al., 2007). The LEF1/TCFs are essential cell fate determination transcriptional factors of the high-mobility group (HMG). LEF/TCFs themselves process little transcriptional activities, rather, LEF/TCF-mediated transcription is tightly controlled by its associated factors (Hurlstone and Clevers, 2002). Previous investigations showed that in the Wnt signaling pathway LEF1/TCFs are transactivated by beta-catenin, which serves as a major dorsalizing signal during the establishment of the dorsoventral axis (Brannon et al., 1997). Besides its role in early patterning formation, excessive activation of LEF1/TCFs by β-catenin has been implicated in malignant transformation of a variety of solitary as well as hematopoietic malignancies (Barker et al., 2000). Given the antagonism between the Xom and beta-catenin in dorsoventral patterning during
early embryogenesis, we have explored the potential role of a human Xom homologue in tumorigenesis.

We searched the EST database and identified a candidate human Xom homologue, Hom-1, also known as Ventx2 or HPX42B (Moretti et al., 2001) (Moreau-Aubry, 2000 #404). As shown in Figure 1 a b, similar to Xom, Hom-1 contains a N-terminal serine/threonine/pralinerich domain, a homeodomain of 61 amino acids, and a C-terminal proline-rich domain. The homeodomain of Hom-1 shares 61% identity and 86% concensus with Xom. A homology plot shows that the overall sequence similarity between Hom-1 and Xom is around 40% with relative higher homology at the HD domain and the C terminal and lower homoloy at the N terminal (figure 1 a, c). Besides structural similarity, using SAGE analysis, we found that the expression pattern of Hom-1 is similar to Xom; both are expressed in eye and germ cells but minimally in other adult tissues. We found that Hom-1, like Xom, is a transcriptional repressor of dorsal gene expression. Thus, on the basis of similarities in structure, function, and pattern of expression, we concluded that Hom-1 is a human homologue of vertebrate Xom. The importance of Xom in cell fate determination during early embryogenesis prompted us to further explore the potential role of Hom-1 in cell fate determination. Given the implication of beta-catenin/LEF/TCF in cancer cell growth, we explored the effects of Hom-1 expression on the proliferation of cancer cells.

Our studies showed that Hom-1 is a nuclear protein. Hom-1 expression exerts strong inhibitory effects on the growth of cancer cells derived from lung and colon. The effects of Hom-1 on cancer cell growth appear to be equally strong in cancer cells that contain wild-type p53 and cancer cells that have a mutated or deleted p53. The effects of Hom-1 on cancer cell growth were assessed both in vitro in tissue culture and ex vivo in nude mice. Because over expression of Hom-1 activates caspase-3, apoptosis may prove to be an underlying mechanism of Hom-1-induced inhibition on cancer cell growth.
Results:

**Hom-1 encodes a nuclear protein**

Structural analysis shows that Hom-1 shares strong homology with Xom (Figure 1a). Hom-1 encodes a polypeptide of 258 amino acids (figure 1b). The homeodomain spans amino acids 91 to 151, is preceded by a serine/threonine-rich region, and is followed by a proline-rich domain. To determine the subcellular localization of Hom in cells, we constructed an expression vector encoding the full-length Hom-1 protein fused in frame with GFP on the N-terminal end (figure 1b). HCT116 cells were transfected with plasmids encoding GFP-Hom-1 or GFP only, subcellular localization of GFP-Hom-1 or GFP was analyzed with a confocal microscopy. While GFP was distributed throughout the transfected cells, GFP-Hom-1 was concentrated in the nuclei of transfected cells, which were stained with propidium-iodide (PI) (Figure 1c). We have further segregated the sub-cellular fractions by density gradient and determined the distribution of GFP-Hom-1 and GFP in different fractions by western blot analysis. We found again that Hom-1 is concentrated in the nuclear fraction, in contrast to the cytosolic and nuclear distribution of GFP (Figure 1d).

**Hom-1 suppresses the growth of human cancer cells through induction of apoptosis**

To explore the potential effects of Hom-1 on the growth of human cancers, we tested the effects of Hom-1 on four cancer cell lines (HCT116 and SW480 cells originated from colon cancers, H460 and H1299 from lung cancers). We also included the human embryonic kidney cell line 293T as a non-malignant control. The effects of Hom-1 on the growth of these cells were determined by transient transfection assay. For all five cell lines, 35-50% of the cells were transfected as indicated by the GFP signal (data not shown). Forty-eight hours post-transfection, cell growth was analyzed
by MTS assay to measure cell metabolism, following manufacturer’s instructions. As shown in figure 2a, compared with GFP, Hom-1 exerts strong inhibitory effects on the growth of the cancer cells (Figs. 2A and 3A). Interestingly, GFP-Hom-1 did not suppress cell growth in the non-cancer 293T cell line (Fig. 2A). Besides using the MTS assay, we have also examined the effects of Hom-1 on the growth of HCT116 cells by microscopic examination. As shown in Figure 2B, GFP and GFP-Hom-1 were expressed after 24 hours post-transfection. When the specimens were re-examined after 96 hours post-transfection, the cell density in the wells that were transfected with GFP-Hom-1 was much less compared to the cell density in the GFP-transfected wells, suggesting that cells expressing GFP-Hom-1 did not survive to 96 hours post-transfection.

After the transient transfection studies, we further investigated the effect of Hom-1 on cancer cell survival in a colony formation assay. Cells were transiently transfected with GFP or GFP-Hom-1; 48 hours later the cells were collected, and positively transfected cells were sorted out using GFP. GFP-positive cells were plated in six-well plates at $1 \times 10^5$ cells per well. Ninety-six hours after plating, colony formation was quantified. As shown in Figure 2c, in comparison with the cells expressing GFP, no colonies were formed in the HCT116 cells or in the H460 cells transfected with GFP-Hom-1. Consistent with its limited inhibitory effects on the growth of 293T cells, the colony formation of 293T cells was minimally affected by the expression of GFP-Hom-1. There was only 20% reduction in colony formation in cells transfected with GFP-Hom1 compared to cells transfected with GFP alone (Figure 2c).

To explore the mechanisms underlying Hom-1-induced growth arrest of cancer cells, we examined the morphology of cells transfected with GFP-Hom-1. As shown in Figure 2d, in comparison with cells transfected with GFP, cells transfected with GFP-Hom-1 appeared to be shrunken and fragmented. When the nuclei of the transfected...
cells were labeled with Hoechst33258, we found that the nuclei of cells transfected with GFP-Hom-1 became condensed and fragmented, a mark of apoptosis (Waldman T, et al. Nature 1996, 381:713). Quantification of the apoptosis showed that, while GFP-Hom-1 induces apoptosis in tested cancer cells, GFP-Hom-1 exerts minimal apoptotic effects on 293T cells (figure 2 e).

Hom-1 induces apoptotic in cancer cells that contain either wild-type or mutated p53

p53 is a critical tumor suppressor gene and is inactivated in about half of tumors as a result of mutations (Vogelstein, 2000 #580). That observation was further confirmed by whole genome sequencing of colon cancers (Selivanova and Wiman, 2007). In addition, p53 mutations occur in ~50% of non-small-cell lung cancers and >70% of small-cell lung cancers (Hollstein et al., 1991). To determine whether the tumor inhibitory effects of Hom-1 is dependent on a functional p53, we examined the effect of Hom-1 on the growth of three cancer cell lines that have either mutations or deletion of p53 (HCT116 p53KO, p53 null H1299, and p53 mutated SW480), by MTS assay, colony formation assay, and morphological examination. As shown in Figure 3, in these cells that lack functional p53, GFP-Hom-1 but not the control GFP significantly suppressed cancer cell growth (Figure 3 a-c). When the nuclei of the Hom-1-transfected p53-defective cells were labeled with Hoechst33258, again we found that Hom-1 induced chromatin condensation and nuclear fragmentation in these p53-defective cells. These data indicate that Hom-1 induced apoptosis in both p53-sufficient and -deficient cancer cells.
**Hom-1 induces Caspase-3 activation**

Caspase activation is the mechanism underlying apoptosis. Caspase-3 has been identified as a key executor of apoptosis in mammalian cells. During apoptosis, caspase-3 is activated through proteolytic cleavage of procaspase-3, which yields an active caspase-3 of 17 kDa and a 12-kDa fragment. Activated caspase-3 in turn fragments, which then targets other key modulators of the apoptotic pathway, such as the nuclear enzyme poly-ADP-ribose polymerase (PARP) (Fernandes-Alnemri T, et al. J Biol Chem 1994, 269:30761). Thus, to explore the mechanism underlying Hom-1 induced apoptosis, we examined the effects of Hom-1 on the activation of Caspase-3. GFP-Hom-1 or control GFP were expressed in HCT116 cells by transient transfection, total proteins were extracted, and caspase-3 activation was analyzed by western blot using an active caspase-3 antibody. As shown in Figure 4, expression of GFP-Hom-1 but not GFP activates the caspase-3. Consistent with activation of caspase-3, expression of Hom-1 in HCT116 cells also leads to the cleavage of PARP, a substrate of caspase-3. Consistent with our observation that Hom-1 induces apoptosis in both p53-sufficient and -deficient cancer cells, we found that expression of Hom-1 induces caspase-3 activation in cells that contain wild-type p53, such as the HCT116 and H460 cells, as well as p53-deficient cancer cells, such as H1299 and HCT116 p53-/- cells.

**Hom-1 represses tumor growth ex-vivo**

Following our investigations of the effects of Hom-1 on the growth of cancer cells in vitro, we went further to determine whether Hom-1 inhibits cancer cell growth in vivo by examining its effects on the growth of HCT116 cells and HCT116p53-/- cells in nude mice. These colon cancer cells were transfected with plasmids encoding either GFP or
GFP-Hom-1. According to the GFP signal, 35-40% of the cells were positively transfected (Figure 5a); 10^6 cells were then injected subcutaneously in the back of nude mice, and the animals were observed up to 21 days. As shown in figure 5 b, both the GFP-transfected HCT116 cells and p53-/- HCT116 cells grew rapidly \textit{in vivo} in the nude mice. In contrast, tumor cell growth was significantly repressed in GFP-Hom-1-transfected cells (Figure 5 b and c). Histological studies of the excised tumor specimens showed that expression of Hom-1 induced apoptosis in tumors, which was revealed \textit{in situ} by TUNEL staining (Figure 5 d). These data indicate that, consistent with its \textit{in vitro} effects, Hom-1 inhibits tumor growth \textit{in vivo} through induction of apoptosis.
Discussion:

Homeobox proteins are essential cell fate determination factors. Our previous studies showed that Xom, an essential vertebrate cell fate determination factor in early embryogenesis, binds to the LEF1/TCF transcriptional factors to exert its essential function during gastrulation. LEF1/TCFs were previously identified as the nuclear mediator of beta-catenin. Besides its role in early embryogenesis, the beta-catenin/LEF/TCF pathway has been implicated in malignant transformation of a variety of cancers, such as colorectal cancer and lung cancers. Given the antagonism between Xom and beta-catenin in cell fate determination during early embryogenesis, we asked whether the human Xom homologue inhibits the growth of cancer cells. In these studies, using methods of reverse genetics, we identified a human Xom homologue, Hom-1, with similarities in structure, function, and pattern of expression. Thus, we have further explored the effects of Hom-1 on the growth of cancer cells originated from colon and lung.

*Hom-1 inhibits the growth of colon and lung cancer cells*

Currently little information is available about the physiological functions of Hom-1, other than its homology to the vertebrate Xom, a essential cell fate determination factor implicated in ventral cell differentiation. Since down regulation of homeobox protein (implicated in cell differentiation) is common in cancer cells (for example, cdx2/4 in colorectal cancer, Nkx3.1 in prostate cancer, KLF 2/4 in colorectal cancer, and Hoxb4 in hematopoietic malignancies (Nunes et al., 2003), we set out to explore the effects of overexpression of Hom-1 on the growth of colon cancer and lung cancer cells. We found that Hom-1 exerts strong growth-inhibitory effects on these cancer cells, as revealed by metabolic labeling (MTS assay), colony formation, and ex-vivo tumor formation assay. It appears that cancer cells are more sensitive to the over expression of Hom-1, since the growth-inhibitory effect of Hom-1 on 293T cells was much smaller
than the effect of Hom-1 on colon and lung cancer cells. The molecular mechanisms underlying the differential response of cancer cells and non-cancer cells to Hom-1 remain unknown. Similar effects were also obtained by treating cancer cells with a DNA decoy that blocks the beta-catenin/LEF/TCF pathway (Seki et al., 2006), possibly related to the previously noted phenomenon of cancer hypersensitivity of cancer addiction to certain genes/pathways (Weinstein, 2000; Weinstein, 2002). Gain-of-function mutations of the beta-catenin/LEF1/TCF pathway have been implicated in the oncogenesis of colorectal cancers. Nevertheless, targeted deletion of beta-catenin blocks the growth of SW48 and DLD1 cells but not of HCT116 cells (Kim et al., 2002). Therefore, the inhibitory effects of Hom-1 on the growth of HCT116 cells suggests that besides its potential effects on beta-catenin activation of LEF/TCF, Hom-1 is likely to affect other critical cell fate determination pathways.

Hom-1 inhibits the growth of cancer cells that contain either wildtype p53 or mutated/deleted p53.

p53 is a critical tumor suppressor gene and is inactivated in about half of tumors as a result of mutations (Vogelstein et al., 2000). P53 plays a pivotal role in regulating cell proliferation and differentiation, serving as a critical link to cell defense mechanisms against carcinogenesis, such as cell cycle control, apoptosis, and senescence. Nonetheless, both p53-dependent and -independent mechanisms have been implicated in tumor suppression, such as in the cases of ARF tumor suppressor genes (Saporita et al., 2007). Therefore, our finding that Hom-1 exerts strong growth inhibitory effects on cancer cells prompts us to examine whether the effects of Hom-1 on the growth of cancer cells depends on a functional p53. Our finding that Hom-1 inhibits the growth of SW48, H1299, and HCT116 P53-/- cells in vitro and ex vivo suggests that the growth inhibitory effects of Hom-1 on cancer cells is not dependent on a functional p53. Given
the high frequency of p53 mutations in cancer cells, our findings suggest the use of Hom-1 in treating cancers that contain p53 mutations.

_Hom-1 activates caspase-3 and induces apoptosis_

Apoptosis plays an essential role in morphogenesis during early development and tumor suppression. Apoptosis can be activated by both the intrinsic mitochondria-mediated pathway and extrinsic death receptor-signal-induced pathways (Debatin, 2004). These pathways converge on the activation of executor caspase 3 to cleave downstream genes to initiate apoptosis, which is marked by chromatin condensation and fragmentation. Our data show that Hom-1 exerts strong growth-inhibitory effects on cancer cells. Both morphological and biochemical data indicated that over-expression of Hom-1 inhibits the growth of cancer cells through activation of caspase and apoptosis. We have yet to determine whether Hom-1 activates the caspase-3 through an intrinsic or extrinsic pathway. Moreover, it remains to be determined whether Hom-1 inhibits cancer cell growth through other mechanisms, such as governing cell cycle progress.
Materials and methods:

Cloning of Hom-1

Hom-1 genome BAC RP13 clone was a generous gift from The Wellcome Trust Sanger Institute, UK. Primer pair (5’ AATTGAATTCAATGCGCCTCTCCTCCTCC3’) and (5’ TTAATCTAGATCATCAAAATGCATCCCCGTCTG 3’) that contain EcoR1 and Xba1 sites were used to amplify the 2.4kb Hom-1 genomic sequence. The PCR product was digested with EcoRI and XbaI, and inserted into CS2 vector. The clone was sequenced, and transfected into 293T cells. The RNA of transfected 293T cells was collected and subjected to RT-PCR with Invitrogen, SuperSript First-Strand Synthesis System for RT-PCR. Poly-A primer was used for RT, Hom-1 primers were used for PCR. The PCR product was cloned into CS2 vector.

Cell Culture, and Transfection

The human colorectal cancer cells, HCT116, SW480, and the human lung cancer cells H460 and H1299 were from America Type Culture Collection (Manassas, VA). The HCT116 (p53⁻⁻) cell line was a gift of Dr. Bert Vogelstein, Johns Hopkins University. All cell lines were maintained at 37°C and 5% CO₂. Cell culture media included McCoy’s 5A (Invitrogen, Carlsbad, CA) for HCT116, HCT116 (p53⁻⁻) and SW480; RPMI-1640 (Invitrogen) for H460 and H1299. The cell culture media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Mediatech, Hemdon, VA). Transfection was done with Lipofectamine 2000 (Invitrogen) following manufacturer’s instruction.
Confocal Microscopy

HCT116 cells were seeded on glass chamber slides and transfected with Hom-GFP expression constructs. Twenty-four hours post-transfection, the cells were fixed with paraformaldehyde in PBS, and counterstained by propidium iodide (PI, Sigma). After four washes in PBS for 5 min each, the slides were mounted and analyzed by confocal microscopy.

Apoptosis and Growth Assay

Cells including attached plus floating cells in the medium were harvested and fixed in a solution containing a final concentration of 3.7% formaldehyde, 0.5% Nonidet P-40, and 10 µg/ml 4′,6-diamidino-2-phenylindole in PBS. Apoptosis was assessed through microscopic visualization of condensed chromatin and micronucleation. At least three independent experiments were carried out for each condition, and a minimum of 400 cells were counted in each measurement.

Cell growth was measured by 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay in 96-well plates (8,000 cells per well) using the CellTiter 96 AQueous One Solution (Promega, Madison, WI) following the manufacturer’s instruction. \(A_{490\ nm}\) was measured using a VERSAmax Tunable Microplate Reader (Sunnyvale, CA). Vehicle-treated cells were as 1 (100%). Each experiment was done in triplicate and repeated at least twice.

Colony Formation Assay

Cells were transfected with Lipofectamine 2000 (Invitrogen) for 24 h in 100mm cell culture dishes according to manufacturer’s instruction. Then, the cells were collected, and sorted according to GFP signal, using a FAC G4 sort flow cytometer (BD Biosciences). These GFP-positive cells were plated in six-well plates at dilutions of
1 × 10^5 cells each well. Cells were allowed to grow for 5-10 days before staining with Crystal Violet (Sigma). All experiments were repeated at least twice, and similar results were obtained in each trial.

**Xenograft Tumors and Tissue Staining**

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Harvard Medical School. Cells were transfected with Lipofectamine 2000 (Invitrogen) for 24 h in 100mm cell culture dishes. Then, the cells were collected, and sorted according to GFP signal, using a FAC G4 sort flow cytometer (BD Biosciences). Xenograft tumors were established by subcutaneous injection of 1 × 10^5 vector-GFP or Hom-GFP positive HCT116 (p53^+/+) or HCT116 (p53^-/-) cells into both flanks of 5- to 6-week-old female athymic nude mice (Simonsen Laboratories, Gilroy, CA). In separated experiments, unsorted transfected cells of 1 × 10^6, including GFP-positive and –negative cells, were also injected subcutaneously into other nude mice to establish an unsorted xenograft tumors mode. Tumor growth was monitored three times a week by a caliper to calculate tumor volumes according to the formula (length x width^2) / 2. The Xenograft tumors tissue was immediately fixed in 10% neutral buffered formalin. The tissues were then embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin (H&E), and then subjected to histological analysis. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done using recombinant terminal transferase (Roche, Indianapolis, IN) and dUTP-Alexa 594 (Molecular Probes) according to the instructions of the manufacturers and counterstained by 4′,6-diamidino-2-phenylindole. All images were acquired with a Nikon TS800 fluorescence microscope using SPOT camera imaging software.
**Cellular Fractionation**

Floating and attached cells were harvested from two 60-cm² dish by centrifugation, re-suspended in homogenization buffer [0.25 mol/L sucrose, 10 mmol/L HEPES (pH 7.4), and 1 mmol/L EGTA], and subjected to 40 strokes of homogenization on ice in a 2-mL Dounce homogenizer. The homogenates were centrifuged at 1,000 x g at 4°C for 10 minutes to pellet nuclei. The supernatant was subsequently centrifuged at 14,000 x g at 4°C for 30 minutes to obtain cytosolic (supernatant) fractions.

**Western Blotting**

Total cell lysates, mitochondrial and cytosolic fractions were purified and separated by 4-20% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) electrophoresis. For active caspase-3 and PARP analysis, total cells were extracted and separated by 4-20% Tris-Glycine Gel (Invitrogen) electrophoresis. Antibodies used include GFP (Santa Cruz Biotech, Santa Cruz, CA), Histone-1 (Ab-1, NeoMarkers, Fremont, CA), and α-actin (Sigma), active caspase-3 (BD Biosciences), and PARP (Cell Signaling Technology, Boston, MA). Hom-1 antibody is custom synthesized by Genemed Synthesis, Inc. with synthesized polypeptides N’ SSPPRGPOQQLSSFGVC C’ and N’ QTSGAREPPQAVSIKEC C’. Appropriate Horseradish peroxidase-conjugated secondary antibodies were used to detect the bound primary antibodies antigen complex and developed with Western Lightning® Western Blot Chemoluminescence Reagent Plus (PerkinElmer, Boston, MA).
Figure 1 Hom-1 encodes a nuclear protein. (A) Alignment of the predicted amino acid sequence of Xom and Hom-1. (B) Schematic of Hom-1 structure. (C) Nuclear location of Hom-1 in transiently transfected HCT116 cells. Plasmids encoding GFP-Hom-1 or GFP were transfected into HCT116 cells. 48 hours post-transfection, the cells were fixed, and the nuclei were labeled by propidium iodide (PI). These cells were then analyzed by confocal microscopy. (D) GFP-Hom-1 or GFP expressing cells were fractionated into cytoplasmic or nuclear fraction through centrifugation. The distributions of GFP-Hom-1 or GFP in each fraction were determined by western blot analysis using an anti-GFP antibody. Histone-1 and β-actin were used as the control for nuclear and pan-cellular distribution marker. Note: GFP-Hom-1 was detected in the nuclear fraction but not the cytoplasmic fraction.
Figure 2

Figure 2 Hom-1 inhibits the growth of human cancer cells and activates apoptosis.

(A) MTT assay of cells transiently expressing Hom-1. Cancer or control cells were transfected with plasmids encoding GFP-Hom-1 or GFP. The growth of vehicle-treated cells was defined as 100%. 293T cells were used as non-cancer controls. (B) Growth of positively transfected cells. 48 hours after transfection, the positively transfected GFP-positive cells were sorted and plated at a dilution of 1 x 10^5 cells per well. 96 hours after plating, the cells were examined with fluorescence microscopy. Note: only a few green cells were found in GFP-Hom-1-transfected wells. (C) Colony formation assay for long-term cell survival. After plating the sorted GFP-positive cells for 7-10 days, the colonies were visualized by crystal violet staining. (D) Apoptotic figures associated with Hom-1 expression. Plasmids encoding GFP-Hom-1 or GFP were transfected into HCT116 cells. 48 hours post-transfection, the cells were harvested, nuclei were stained with Hoechst 33528, and cells were examined with phase contrast and fluorescence microscopy. Apoptotic cells displayed characteristic condensed chromatin and fragmented nuclei. (E) The percentage of apoptotic cells was calculated by counting a minimum of 400 cells in each determination, and the experiment was repeated twice with identical results. Values are mean ± SD, *P<0.01 v.s. vector-transfected cells.
Figure 3. Hom-1 inhibition of cancer cell growth and induction of apoptosis in cancer cells that contain no functional p53. Cancer cells that contain mutated p53 (SW48), p53 null mutations (H1299), or p53 deletions (HCT116 p53-/-) were transfected with plasmids encoding GFP-Hom-1 or GFP, and the effect of Hom-1 expression on the growth of these cancer cells was assessed. (A) MTT assay of cells transiently expressing Hom-1. Cancer or control cells were transfected with plasmids encoding GFP-Hom-1 or GFP. The growth of vehicle-treated cells was defined as 100%. (B) Growth of positively transfected cells. 48 hours after the transfection, the positively transfected GFP-positive cells were sorted and plated at a dilution of $1 \times 10^5$ cells per well. 96 hours after the plating, the cells were examined with fluorescence microscopy. Note: few green cells were found in GFP-Hom-1-transfected wells. (C) Colony formation assay for long-term cell survival. After plating of the sorted GFP-positive cells for 7-10 days, the colonies were visualized by crystal violet staining. (D) Apoptotic figures associated with Hom-1 expression. Plasmids encoding GFP-Hom-1 or GFP were transfected into HCT116 cells. 48 hours post-transfection, cells were harvested, nuclei stained with Hoechst 33528, and the cells examined with phase contrast and fluorescence microscopy. Apoptotic cells displayed characteristic condensed chromatin and fragmented nuclei. (E) The percentage of apoptotic cells was calculated by counting a minimum of 400 cells in each determination, and the experiment was repeated twice with identical results. Values are mean ± SD, *$P<0.01$ v.s. vector-transfected cells.
Figure 4. Overexpression of Hom-1 activates caspase-3. Plasmids encoding GFP-Hom-1 or GFP were transfected into HCT116 cells. 48 hours post-transfection, total cellular proteins were extracted, and caspase-3 activation was assessed by western blot using an active caspase-3 antibody. The activation of caspase-3 was also determined by cleavage of PARP, a caspase-3 substrate, using a specific PARP antibody. β-actin was used as the loading control.
Figure 5. Hom-1 inhibits tumor growth and induces apoptotic cell death ex vivo.

(A) HCT116 or HCT116 p53-/- cells were transfected with plasmids encoding GFP or GFP-Hom-1. 35-40% of the cells were positively transfected as indicated by the GFP signal. (B) Top panel depicts the tumor-bearing nude mice, and the lower panel shows the tumors excised from the nude mice. (C) Tumor growth was significantly repressed in both GFP-Hom-1-transfected wildtype HCT116 cells and HCT116 p53-/- cells. Values are mean ± SD, n=5 per group, GFP-Hom-1 vs. GFP-treated group, *P<0.01. (D) The xenograft tumor tissue was fixed, embedded, and sectioned. Apoptosis was analyzed by TUNEL staining (red) with DAPI counterstaining (blue). Tumor histology was analyzed by H&E staining.
References:


Summary and Conclusion

Deregulated signal transduction of early embryonic cell fate determination pathways are often implicated in pathological conditions in adult life, such as neogenesis and birth defects. Dissecting the molecular mechanism of signal transduction during early embryonic development therefore may provide critical information for the prevention and treatment of neoplastic diseases. I have focused my efforts on the molecular mechanism of BMP4/Xom signaling during early vertebrate embryogenesis. More knowledge of this signaling pathway has emerged from this project.

BMP4 is an essential ventral cell fate determination pathway of early embryogenesis, and counteracts the dorsal signaling of the Wnt/beta-catenin/LEF/TCF. How BMP4 and Wnt/beta-catenin/LEF/TCF pathway integrate during the establishment of dorsoventral axis is not well understood. Xom is the major transcriptional mediator of the BMP4 pathway during early vertebrate embryogenesis. Therefore, to determine the molecular mechanism of integration of the BMP4/Xom signal and the Wnt/beta-catenin/LEF/TCF signal in cell fate determination, I examined the effects of Xom on LEF/TCF-mediated transcription. I found that Xom bound to LEF/TCFs and transactivated LEF/TCF mediated transcription. I also found that Xom/LEF/TCF signaling was essential for the generation of BMP4 signal and normal progress of gastrulation. These findings placed the LEF/TCFs, the previously defined dedicated transcriptional partners of the Wnt/beta-catenin pathway, as the site of convergence of Xom and beta-catenin, the two antagonizing cell fate determination factors. My findings led to a model of competitive between the BMP4/Xom and Wnt/beta-catenin pathways during patterning formation.
Previous studies showed that both Xom and beta-catenin are unstable proteins whose abundances are controlled by ubiquitin-mediated proteolysis. The findings that both Xom and beta-catenin converge on the common LEF1/TCF transcriptional factors for their combined effects on cell fate suggest that relative stability of Xom and beta-catenin should play essential role in determining their integrated effects on cell fate determination. Thus, I have further explored the regulatory mechanism of Xom proteolysis during early embryogenesis. Using an in vitro ubiquitination assay, I found that Xom contained a degron whose function was dependent on Ser140/144. I obtained evidence showing that Xom Ser140/144 phosphorylation was required for its interaction with β-TRCP, a component of Xom E3 ligase. I found that the Xom Ser140/144 phosphorylation pattern correlated with Xom degradation pattern in the staged embryonic extracts, suggesting that phosphorylation of Xom Ser140/144 plays a regulatory role in controlling Xom stability.

The Wnt/beta-catenin signaling has been implicated in the pathogenesis of a variety of cancer syndromes, such as colon cancers. Xom antagonizes beta-catenin during early embryogenesis. Therefore, following my identification of Xom intersection on the Wnt/beta-catenin pathway, I went on to study a human Xom homologue, Hom-1 and explored its effects on the proliferation of cancer cells. I found that Hom-1 exerted strong inhibitory effects on the several tested cancer cells, such as colon and lung cancers, both in vitro and ex-vivo in nude mice. Moreover, I found that the growth inhibitory effect of Hom-1 on cancer cell growth was not dependent on a functional p53, a prominent tumor suppressor gene that is frequently mutated in cancers.
In summary, through developmental modeling, I have identified a novel signaling pathway of the Xom/LEF/TCF. I found that the Xom signaling was regulated by Ser140/144 phosphorylation controlled proteolysis of Xom. Besides its essential function in early embryogenesis, I found that human Xom homologue exerted strong growth inhibitory effects on cancer cell growth. Future work will be directed towards further exploration of the physiological function of Xom/LEF/TCF signaling in early embryogenesis, identification of the Xom-kinases, and the evaluation of Hom-1 as a novel putative tumor suppressor gene.
Appendix

Experimental Material and Methods:

A. Promoter Luciferase assay

Rationale: Previous studies have defined the LEF1/TCF factors as the sole transcriptional partners of Wnt/β-catenin, therefore, the LEF1/TCF promoter-luciferase reporter activity has generally been regarded as an indicator of Wnt/β-catenin activities. As such, to monitor the Wnt signaling, a TOPflash construct was developed, in which three copies of the minimal essential TCF/LEF binding motif were fused to the reporter gene Luciferase, whose activities presumed to reflect the transcriptional activation of the LEF1/TCFs.

Method: For the cellular Luciferase assays, 2x10^5 cells were split into 12-well cell culture plates. The cells were incubated at 37°C, 5%CO2, 95% humidity for 24 hours. Each well of the cell was transfected with 3 µl of liposome transfection reagent (TransIT1, Mirus), 1 µg of DNA plasmid of selected genes, and 0.3 µg of TOP or FOP plasmid. 48 hour after the transfection, cells were washed with PBS, lysed with 1 x cell lysis buffer (Promega cell lysis buffer), scrapped, collected into a microcentrifuge tube, incubated on ice for 30 minutes, centrifuged at 12000x g for 15 seconds, and transferred to a new tube. 20 µl of the cell lysate was mixed with 100 µl of Luciferase Assay reagent (Promega, Luciferase Assay System), and the activity was read on TR717 Microplate luminometer (Applied Biosystem) using WinGrow software.

For the Xenopus embryos Luciferase assay, 0.4 ng of in vitro transcribed RNA of the selected genes and 100 pg of TOP or FOP plasmid were co-injected into the embryos at the two-cell stage. The embryos were cultured at 18°C for ~12 hours till reaching stage
9. 5 embryos were collected and the proteins were extracted in 100 mM KCl, 1 mM MgCl$_2$, 100 nM CaCl$_2$. 1 µl of the embryo extraction sample was mixed with 100 µl of Luciferase Assay reagent, and the activity was read on a TR717 Microplate luminometer.

**B. Immuno-precipitation:**

Rationale: To prove our hypothesis that Xom and LEF/TCF form a complex intracellularly, we transfected myc-tagged Xom plasmid into HCT116 cell line, a colon cancer cell line with high level of endogenous expression of TCF4. If Xom could form a complex with TCF4, myc-tagged Xom will be pulled down together with TCF4 by TCF4 antibody coded agarose beads. This co-precipitation will be detected by Western blot with anti-myc antibody.

Method: 2X10$^6$ of cells were lysed with 1x cell lysis buffer (promega) with 1x protease inhibitor reagent (Roche), incubated on ice for 30 minutes, briefly sonicated, and spinned at 12000g for 5 minutes at 4°C. The supernatants were pre-cleaned with 20 µl nude protein G plus-agarose Beads (Santa Cruz Biotechnology) and 2 µg of corresponding serum for 2 hours at 4°C. 20 µl of the Protein G plus-agarose beads was pre-labeled with 2 µg of corresponding antibody at 4°C for 2 hours. The supernatants were then mixed with 20 µl of pre-labeled 20 µl agarose beads, and incubated at 4°C overnight with shaking. The beads were washed four times with 1 x PBS + 0.2% NP$_{40}$. The beads were then mixed with 2 x sample buffer, boiled at 95°C for 5 minute, briefly spinned, and subjected to Western blot analysis.
C. In vitro GST pull-down assay

Rationale: To prove our hypothesis that Xom binds to LEF-1 directly, we used BL-21 bacterial strain to express GST-tagged Xom protein, and GST-tagged Xenopus LEF-1 protein. Meanwhile, we also expressed $^{35}$S labeled Xom and LEF-1 using In Vitro Transcription and Translation System. If the two proteins can bind to each other, when $^{35}$S labeled Xom was incubated with GST-tagged LEF-1 or $^{35}$S labeled LEF-1 was incubated with GST-tagged Xom, they will be pulled down together by Glutathine Sepharose 4B beads (GST protein binds tightly to Glutathine sepharose beads). The coprecipitation will be detected by autoradiography.

Method: 1) preparation of GST tagged proteins: Selected genes were cloned into PGEM 4T-3 vector, and transformed into BL21-Codon Plus competent cells (Stratagene). The individual clones were grown up in LB/Ampicilline culture medium. The protein expression was induced with 100 mM IPTG. The GST fusion proteins were purified with Glutathione Sepharose 4B beads following manufacture instructions (Amersham). The proteins were desalted and concentrated with Amicon Ultra Centrigual Cellulose columns (Millipore). The expression of GST fusion proteins were detected by western blot with anti-GST antibody. Bicinchoninic Acid method was used to determine the fusion protein concentration (BCA Protein Assay kit, Pierce). 2) Preparation of $^{35}$S labeled proteins: Proteins were transcribed and translated using TNT Coupled Reticulocyte Lysate System (Promega). 1 µl of $^{35}$S labeled methionine (Perkin Elmer, NEG 772002MC) was added to the reaction mixture for protein labeling. 3) In vitro pulldown assay: 5 µg of GST fusion protein and 5 µl of $^{35}$S labeled IVT proteins were mixed with 20 µl of 50% Glutathine Sepharose 4B beads in 500 µl of binding buffer (50 mM Tris PH 8.0, 150 mM KCL, 5
mM MgCl₂, 0.2% NP₄₀). The mixture was incubated at 4°C for 3 hours, and the beads were washed four times with 1x PBS + 0.2% NP₄₀. The beads were then mixed with 2x sample buffer, boiled at 95°C for 5 minute, briefly spun, and subjected to SDS-PAGE gel electrophoresis. The gels were vacuum dried, and subjected to autoradiography.

D. In vitro transcription for RNA preparation, and RNA injection to Xenopus embryos, and phenotype recording.

Rationale: To further explore the in vivo importance of the complexation of Xom and LEF1, mRNA of Xom molecule and Xom mutants were prepared and micro injected into xenopus embryo. The phenotypes of over expression of Xom protein and mutant Xom proteins were closely recorded in a timely fashion.

Method: mRNAs were prepared with mMessage mMachine in vitro transcription Kits for synthesis of capped RNAs (Ambion, cat # AM1340) following manufacture instruction. mRNAs were purified with Rneasy mini-kit (Qiagen, Cat # 74104). The quality and concentration of the RNAs were measured with OD₂₆₀/OD₂₈₀ and gel electrophoresis. ~20 ng of mRNA were injected into the developing xenopus embryos using PLI-100 picoliter injector (Medical System Corp.).

E. RT-PCR and Quantitative real-time PCR

Rationale: RT-PCR (Reverse transcriptase-polymerase chain reaction) is a highly sensitive technique for the detection and quantization of messenger RNA. RT-PCR consists of two steps. The first step is to synthesize cDNA (complementary DNA) from mRNA by Reverse Transcriptase. The second step is to amplify a gene specific cDNA by
PCR (polymerase chain reaction). During the second step, direct detection of specific PCR product could be monitored by measuring the increase of fluorescence caused by the binding of Syber green dye to double strained DNA. Xom is a transcription factor that could regulate the transcription of its downstream genes. RT-PCR is used here to analyze the function of Xom.

Method: 5x10^6 cells were used for RNA extraction. 3 Xenopus embryos were used for RNA extraction. The samples were suspended in 1 ml of Trisol (Invitrogen, Cat # 15596-026). The RNA were extracted with 200 ul Chloroform twice, and precipitated with 600 µl isopropyl alcohol. The pellets were washed with 75 % ETOH. 2 µg of RNA was used for the RT experiment (Invitrogen, SuperSript First-Strand Synthesis System for RT-PCR, cat #12371-019). The RT products were 10 x diluted, and subjected to real time PCR using LightCycler System and LightCycler FastStart DNA Master SYBR Green I (Roche Life Science) according to the manufacturer’s instructions. The relative level of the gene expression was calculated by the formula: relative gene expression = 2^{\Delta C_d} (\Delta C_d=cycle of the specific gene-cycle of the reference Histone-4 gene). Each sample was carried out in triplicates. The data was presented as mean + Standard Deviation.

F. In vitro kinase assay, gel electrophoresis, in gel digestion, sample preparation for Mass spectrometry, and FT-MS/MS

Rationale: Xom is an unstable protein, and serine 144 and serine 140 are important sites for Xom phosphorylation from genetic studies. To further explore the exact site of phosphorylation, Mass spectrometry is used to analyze the site of phosphorylation.
phosphorylation. A mass shift of 80 Dalton could be detected when a phosphor-group is added to the serine.

Method: 5 µg of GST Xom fusion protein was mixed with Xenopus embryonic extract 5 µl, 2 µl 1 mM ATP and 20 mM HEPES buffer in total volume of 25 µl. The mixture was incubated at 37°C for 2 hours, and subjected to SDS-PAGE separation. The band with the right molecular weight was cut out, and soaked in 200 µl 50% ACN/50% 50 mM ammonium bicarbonate solution 5 minutes. The samples were spinned briefly, changed to the same ACN solution, and left overnight. The pellets were washed with 300 µl ACN solution, and speed vacuum dried at 37°C for 1 hour. Trypsin (~1 µg/band) solution in 50mM ammonium bicarbonate was added to each band. The samples were incubated at 37°C overnight. The gel pellets were extracted twice with 5% formic acid/50%Acetonitrile twice, and speed vacuum dried. Liquid chromatography tandem MS (LC-MS/MS) was performed by using a 7-T LTQ FT hybrid mass spectrometer (Thermo Electron). The digested peptides were loaded onto the resolving column with a capillary auto-sampler binary HPLC pumps with an in-line flow splitter. 17 cm of Reverse-phase Magic C18 material (5 µm, 200Å, Michrom Bioresources) was used in the resolving column. Ten LTQ MS.MS spectra were acquired per cycle in a data-dependant fashion from a preceding Fourier transform MS scan (400-1800 m/a at 100,000 resolution with dynamic exclusion).
G. **In vitro phosphorylation assay**

Rationale: Mass spectrometry is a definite measure to identify the phosphorylation site on a protein. But a negative reading may because of the lost of the phosphorylated group during the preparation process, or may because of the low concentration of the phosphorylated species. In vitro phosphorylation assay is a relative more sensitive measure with less specific site information. In my experiment, in order to find out whether serine 140 or serine 144 or both is phosphorylated, I prepared two polypeptides, one with 140 serine mutated to Analine, one with 144 serine mutated to Analine. The two peptides were incubated with embryonic extract with $^{32}$P labeled ATP. The attachment of $^{32}$P labeled ATP to the peptide could be detected by autoradiography.

Method: 1µg of GST fusion protein was mixed with Xenopus embryonic extract 5 µl, 2 µl 1 mM $^{32}$P labeled ATP and 20 mM HEPES buffer in total volume of 25 µl. The mixture was incubated at 37ºC for 2 hours, and subjected to SDS-PAGE separation. The SDS-PAGE gel was dried and subjected to autoradiography.

H. **Cell Immuno-staining.**

Rationale: To characterize the cellular localization of Hom-1, Myc tagged Hom-1 plasmid was transfected into cell lines. Mouse Anti-Myc antibody (first antibody) will recognize myc-hom-1 and bind to it. FITS-labeled anti mouse antibody ($2^{nd}$ antibody)
will bind to the 1st antibody, which will be imagined by fluorescence CoFocal microscope.

Method: 1X 10^5 cells were plated onto Microscope Cover Glass (Fisher) in 24-well cell culture plate. Twenty-four Hours after plating, the cells were transfected with 1 μl TransIT-1 lipotranfectamine reagent (Mirus) and 0.5 μg of plasmid DNA. 36 hours after transfection, the cells were fixed in 2% Paraformaldehyde at room temperature for 2 hours. The cells were incubated with BSA in 1 X PBS (2 mg/ml) + 0.1% triton for 20 minutes, washed with 1 X PBS, and incubated with 2 ng of first antibody at room temperature for 2 hours. After 2 X PBS wash, the cells were incubated with FITS-labeled 2nd antibody for 2 hours. The microscope cover glasses were mounted onto microscope glass slide using Prolong antifade kit (Molecular probe).

I. Cell proliferation and viability assay.

Rationale: To explore the cellular function of Hom-1 (Xom human homolog), I express the Hom protein in varies cancer cells. The cell proliferation rate and viability were measured by MTS assay. In the assay, the cancer cells will be transfected with GFP vector plasmid or GFP HOM-1 plasmid. The GFP positive cells will be sorted out and plated onto 96 well plates. 48 hrs after the plating, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) will be added into the culture medium for 3 hours. the cell viability will be measured by reading the 490 nm absorbance. MTS is readily bioreduced by metabolic active cells into a formazan
product, which could be measured by the amount of 490 nm absorbance. The quantity of formazoan products is directly proportional to the number of the metabolic active living cells in culture.

Method: 5 x 10^6 Hct 116 Cells were plated onto 10 cm cell culture dish. 24 hours after, the cells were transfected with 20 µl of TrsnIT-1 (Mirus) and 10 µg of indicated plasmid DNA with GFP tag. 36 hours after the transfection, the GFP positive cells were sorted out by FAC G4 sort flow cytometer (BD Biosciences). 1 x 10^5 cells were plated onto 96 well plate, and cultured in cell growth medium. Cell viability was measured with CellTiter 96 Aqueous No-radioactive cell proliferation assay kit (Promega). Each transfection was repeated four times to reduce the experimental variations. The data was presented as mean + SD.

J. Nude mice injection, Xenograft Tumors and Tissue Staining

Rationale: A nude mouse is a mouse mutant that has deteriorated or removed thymus gland, resulting an inhibited immune response. Those mice have no body hair in appearance, which gives them the nickname “nude”. Because of their immune deficiency, they are used widely in tumor xenograft experiments, as they can’t generate immune rejections. In my experiment, in order to find out the function of Hom-1 in tumor genesis, we transfected tumor cells with GFP-Hom-1 and GFP-only plasmid. The GFP positive cells will be sorted out and xenografted subcutaneously (S.C.) into nude mice. The tumor growth of the mice group with GFP Hom-1 transfected cell grafts will be compared with that of the mice group with GFP only transfected cell grafts.
Method: All animal experiments were approved by the Institutional Animal Care and Use Committee at the Harvard Medical School. The cells were transfected with vector-GFP or Hom-GFP plamid using Lipofectamine 2000 (Invitrogen) on 10 cm cell culture dishes. The cells were collected, and sorted using a FAC G4 sort flow cytometer (BD Biosciences). Xenograft tumors were established by s.c. injection of $1 \times 10^5$ vector-GFP or Hom-GFP positive HCT116 cells into both flanks of 5- to 6-week-old female athymic nude mice (Simonsen Laboratories, Gilroy, CA). Tumor growth rate was monitored twice a week by calipers to calculate tumor volume according to the formula $(\text{length} \times \text{width}^2) / 2$. The Xenograft tumors tissue was immediately fixed in 10% neutral buffered formalin. The tissues were then embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin (H&E), and subjected to histological analysis. Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was done using recombinant terminal transferase (Roche, Indianapolis, IN) and dUTP-Alexa 594 (Molecular Probes) according to the instructions of the manufacturers and counterstained by 4',6-diamidino-2-phenyldole.