INCENP TRANSLATED DURING OOCYTE MATURATION IS A MATERNAL FACTOR OF XENOPUS LAEVIS DEVELOPMENT

By

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Abstract

During vertebrate oocyte maturation, the chromosomes progress to and arrest at metaphase of meiosis II in preparation for fertilization. This process includes emission of the first polar body. The second polar body is emitted after fertilization. A number of proteins are accumulated during oocyte maturation. Inhibition of this *de novo* translation does not appear to affect the progression of meiosis during oocyte maturation. The role of these pools of proteins has yet to be elucidated. Curiously, several of the upregulated proteins are key players in mitosis, including INCENP, a subunit of the chromosome passenger complex implicated in chromosome segregation and cytokinesis. During early stages of development in *Xenopus laevis*, the embryo cycles through mitosis, also known as embryo cleavage, every 30min with little to no time for transcription/translation. Our goal is to determine if the *de novo* translation of these mitotic proteins during oocyte maturation has a role in early embryogenesis. We used morpholino oligonucleotides antisense to INCENP mRNA ($INCENP_{morpho}$) to inhibit *de novo* translation during oocyte maturation. Using confocal imaging and the host transfer technique, these injected oocytes were matured, fertilized and assessed for developmental competency. $INCENP_{morpho}$ and a control morpholino ($ctrl_{morpho}$) had no discernable effect on 1^{st} or 2^{nd} polar body emission. Whereas $ctrl_{morpho}$ embryos developed normally, $INCENP_{morpho}$ embryos did not cleave. Thus, *de novo* translation of INCENP during oocyte maturation is necessary for embryogenesis. Specifically, accumulation of INCENP and other mitotic proteins during oocyte maturation may be a common strategy in this species to prepare for the rapid and synchronous mitoses during early embryogenesis.

Acknowledgments

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List of Abbreviations

ART	artificial reproductive technologies
AS	anti-sense
Aur-A	Aurora A
cAMP	cyclic adenosine monophosphate
CG	cortical granule
CPC	chromosomal passenger complexe
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
$ctrl_{morpho}$	control morpholino oligonucleotide
ER	endoplasmic reticulum
GFP	green fluorescent protein
GSH	glutathione
GV	germinal vesicle
GVBD	germinal vesicle breakdown
INCENP	inner centromere protein
$INCENP_{morpho}$	inner centromere protein morpholino oligonucleotide
IVF	*in vitro* fertility
OCM	oocyte culture medium
MBE	Musashi binding element
MBS	modified Barth's solution
MPF	maturation promoting factor
mRFP	red fluorescent protein
MS222	ethyl 3-aminobenzoate methanesulfate salt
NT	neural tube
NTC	neural tube closure
NTD	neural tube defects
PB	polar body
Pcm-1	pericentriolar material-1
RCC1	regulator of chromosome condensation 1
ROS	reactive oxygen species
SC_{morpho}	standard control morpholino oligonucleotide
TCS	translational control sequence
ZGA	zygotic genome activation

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INCENP TRANSLATED DURING OOCYTE MATURATION IS A MATERNAL FACTOR OF XENOPUS LAEVIS DEVELOPMENT

Leblond, Geoffrey

Introduction

The development of an organism is a very complex process. It all begins with the creation of the embryo via a process called embryogenesis. This first stage of vertebrate life contains many steps in itself, which can be divided into five stages: cleavage, morulation, blastulation, gastrulation and organogenesis. After fertilization of the female egg (also called a mature oocyte) by the male sperm, the egg becomes what is called a zygote, initiates mitosis and begins to cleave (divide). This early cleavage state of the embryo defines the morula stage, and results in a mass of undifferentiated cells ready for the next stages of embryogenesis. Although the duplication of cells can seem like a simple process, morulation is much more complex. The zygotic genome that was created by the fusion of the male and female genomic material must begin to transcribe zygotic mRNA. In turn, these zygotic mRNAs are translated into zygotic proteins and begin to control development of the embryo. This genetic reprogramming is referred to as the "zygotic genome activation" (ZGA). Since the sperm contributes few cellular components (mRNA, proteins, macromolecules; Sutovsky and Schatten, 2000), the embryo is, for a time, composed of factors originating almost entirely from the female oocyte and its genome. These components are called "maternal factors". Consequently, the proper growth and maturation of a healthy oocyte is a critical step in the development of an organism.

The study of oocyte health is a focus in assisted reproductive technologies (ART), such as *in vitro* fertility (IVF) treatments. Certain markers of a healthy oocyte have been identified and used to grade oocyte health (Combelles et al., 2002; Watson 2007). However, these markers are based on ultra-structural and gross physiological properties of an egg. This remains superficial and only gives a limited impression of the quality of an oocyte. There is a need for a greater understanding of the biochemical make-up of a healthy oocyte. The purpose of the present paper was to identify and characterize novel maternal factor proteins.

1. Our model, Xenopus laevis

Xenopus laevis (African clawed frog) was used as a model of vertebrate oocyte maturation and development. This model was chosen primarily because of the ease of manipulation and culture of frog oocytes and embryos. The comparatively long period of transcriptional silence in early embryogenesis is an additional advantage because it provides a larger window of opportunity to study the function of maternal factors.

The fully-grown *Xenopus laevis* oocyte is 1.4 mm in diameter. The large size of the oocyte makes it quite robust. Oocytes, as well as embryos, are easily manipulated with forceps and can survive repeated microinjections. Oocytes also require minimal medium for culture and development. This characteristic can best be seen in the embryo, which can be cultured in as simple an environment as de-chlorinated tap water. It is also possible to recover a large number of oocytes and embryos from a single frog. The frog ovary contains thousands of fully-grown oocytes that can be quickly isolated for use in experiments. These various

physical properties of the *Xenopus* oocyte and embryo make it an ideal model for reproductive and developmental biology studies.

The particular development of the *Xenopus* embryo provides additional benefits for studies of embryogenesis. During early embryogenesis, there is almost complete absence of transcription of the zygotic genome. Although there is some evidence of transcription from 1 and 2-cell embryos (Golbus et al., 1973), the bulk of transcription initiation occurs during ZGA (Newport and Kirschner, 1982; Braude et al., 1979; Braude et al., 1988; Plante et al., 1994). Because of this critical change in the transcriptome, it is expected that the proteome will observe a similar change. Not surprisingly, ZGA is marked by a noticeable change in polypeptide expression profiles on polyacrylamide gels (Braude et al., 1988). This indicates a significant change in the biochemical makeup of the embryo, both in the transcriptome and the proteome. Before ZGA, the embryo must rely on maternally accumulated factors, termed "maternal factors", to direct embryogenesis. The timing of the ZGA varies between species. The ZGA occurs at the 1 to 2-cell transition in mice (Braude et al., 1979), at the 4 to 8-cell transition in humans (Braude et al., 1988) and at the 8 to 16-cell transition in cows (Plante et al., 1994). In *Xenopus laevis* however, this transition occurs after the 4000-cell stage (Newport and Kirschner, 1982). This means that prior to fertilization, the *Xenopus* oocyte must stockpile maternal factors that will allow for 12 rounds of mitosis, compared to other species that must only stockpile for anywhere from one to four rounds of mitosis.

Considering the physical properties of the *Xenopus laevis* oocyte and embryo, and the fact that the early embryo displays a lengthy period of transcriptional silence, this species

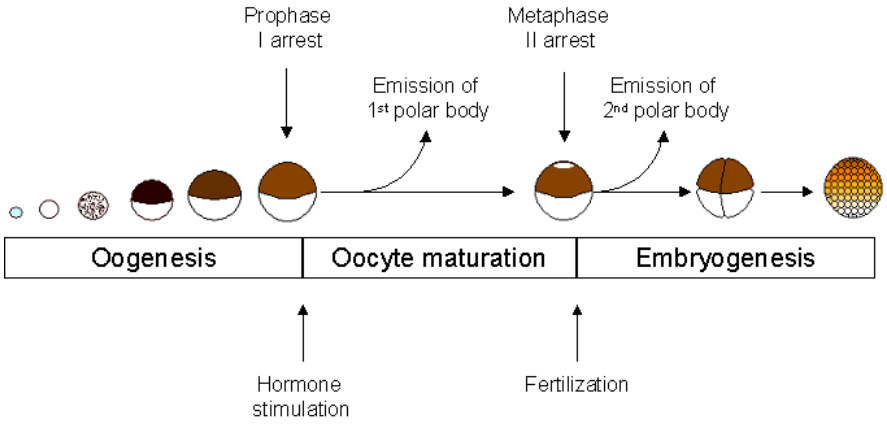
provides an excellent system to identify and analyze maternal factors of vertebrate development.

2. Maturation of the Xenopus laevis *oocyte*

The fully-grown, immature *Xenopus laevis* oocyte is opaque and has three distinct zones that are visible on the cell surface. (Figures 1, 4) The "top" of the oocyte is defined as the animal hemisphere and is darkly pigmented, due to pigment granules at the cortex. The "bottom" of the oocyte is referred to as the vegetal hemisphere, which is beige or white. Between both hemispheres is found the equatorial band, which is a region devoid of pigment. In an immature oocyte, the nucleus, found in the animal hemisphere, is still intact and the chromosomes are arrested in late prophase of meiosis I. The immature oocyte can then be triggered by hormones to progress through oocyte maturation. This generates a mature egg that is ready to be fertilized.

In vivo, the pituitary glands release gonadotropic hormones that circulate through the body and eventually reach the ovary, where they stimulate production of steroid hormones. These steroids, such as progesterone, are produced by the follicle cells that surround the oocytes (Fortune et al., 1975). All of the biochemical pathways that lead to progesterone-initiated oocyte maturation have not yet been elucidated, however, many elements of the pathways have been discovered. Progesterone is known to lower the levels of cyclic AMP (cAMP) in the oocyte by inhibiting adenylate cyclase (Sadler and Maller, 1981). This occurs through two mechanisms: a novel plasma membrane-bound progesterone receptor (Zhu et al., 2003) and the classical progesterone receptor (Bayaa et al., 2000). The decrease in

Figure 1: Life cycle of the oocyte. Oogenesis generates an immature, fully grown oocyte arrested at the prophase stage of meiosis I. After stimulation with hormones, the oocyte resumes meiosis, completes the first meiotic division and arrests as a mature oocyte (egg) in metaphase of meiosis II. Fertilization of the egg triggers the second division of meiosis and subsequent embryogenesis. Image based from Ferrell, 1999.


Prophase
I arrest
Metaphase
II arrest
Emission of
1st polar body
Emission of
2nd polar body
Oogenesis
Oocyte maturation
Embryogenesis
Hormone
stimulation
Fertilization

cAMP levels results in the translation of Mos, a dormant mRNA that is accumulated during oogenesis (Gebauer and Richter, 1997). Through a series of protein kinase cascades, Mos translation in *Xenopus* oocytes leads to the activation of the maturation promoting factor (MPF) and release of the prophase I arrest. The MPF protein complex is a cell cycle regulator and its activation triggers the entry into metaphase. It is composed of cyclin B and its associated kinase cdc2 (Lohka et al., 1988). The primary physiological event in the oocyte after progesterone stimulation is germinal vesicle breakdown (GVBD), or dissolution of the nuclear membrane. In oocytes, the nucleus is sometimes referred to as the germinal vesicle (GV). Consequently, an immature oocyte, whose germinal vesicle is still intact, is referred to as a "GV oocyte". A mature egg, whose germinal vesicle has broken down, is called a "GVBD oocyte/egg".

2.1. Progression through meiosis during oocyte maturation

Meiosis is composed of two rounds of cell division: meiosis I and II. The most obvious change that occurs inside the oocyte during oocyte maturation is the transition from meiosis I to meiosis II. (Figure 1) By foregoing DNA replication between these two rounds, meiosis reduces the ploidy, or number of chromosomes, of the cell by a factor of 2. Meiosis also occurs in the male gamete (the sperm) so that when both gametes' nuclei fuse, they create a zygotic genome with the proper ploidy number. In the case of *Xenopus laevis*, each parent contributes 18 (n) chromosomes, resulting in a 36 (2n) chromosome offspring.

Oocyte maturation begins after stimulation with progesterone. Here, the immature oocyte resumes meiosis from its prophase I arrest. This begins with GVBD and alignment of the

chromosomes to form the first metaphase plate of meiosis, where homologous chromosomes are paired. After separation of these pairs in anaphase I, half of the genetic material is expelled from the oocyte in the form of a polar body (PB). This process is called PB emission, and is an extreme form of asymmetrical cytokinesis. The tiny PB contains only discarded genetic material, allowing for most of the organelles and cytoplasmic factors to remain in the oocyte to support development of the embryo. This difference in size is most notable in the *Xenopus laevis* oocyte. The oocyte can reach 1.4 mm in diameter, whereas both polar bodies that are extruded from the oocyte are only 0.02 mm in diameter (Ma et al., 2006).

Following emission of the first PB, the oocyte arrests at metaphase of meiosis II, awaiting fertilization. Fertilization of this now mature oocyte (also referred to as an "egg") releases the second meiotic arrest, resulting in the separation of sister chromatids, emission of the second PB and, eventually, embryogenesis. Although meiosis is the most characterized process that occurs during oocyte maturation, it is certainly not the only event that takes place inside the oocyte. Protein translation and changes in organelle structure and localization also occur during this time.

GVBD is one of the first events that occur during oocyte maturation. It has long been known that protein translation is necessary for GVBD and oocyte maturation (Wasserman and Masui, 1975). The translation of a specific protein, Mos, was discovered to trigger GVBD and oocyte maturation (Reviewed in Gebauer and Richter, 1997). Inhibition of translation of Mos mRNA inhibits oocyte maturation (Sagata et al., 1988) and injection of Mos protein

triggers oocyte maturation (Yew et al., 1992). Accordingly, proper translation of Mos is critical to oocyte maturation.

Regulation of translation during oocyte maturation is precise and complex (MacNicol and MacNicol, 2010). There are two major classes of mRNA translated during oocyte maturation. Originally termed class I and class II (Ballantyne et al., 1997), they are now called "early" and "late" mRNA, respectively (MacNicol and MacNicol, 2010). The classification is in reference to the time during which each mRNA is translated. Mos, for example, is translated during the early stages of oocyte maturation and is involved in the initiation of GVBD and oocyte maturation itself (Gebauer and Richter, 1997). Therefore, it is considered an "early" mRNA. On the other hand, cyclin B is an example of a "late" mRNA, it is translated after initiation of GVBD and is involved in the progression from MI to MII (Hochegger et al., 2001). The temporal control of these, and many other, mRNA is mediated by polyadenylation, through 3' untranslated region (3'-UTR) binding elements. The major binding proteins are Musashi and cytoplasmic polyadenylation element binding protein (CPEB) which promote early and late mRNA translation, respectively (MacNicol and MacNicol, 2010).

Mos and cyclin B are two examples of proteins critical to the initiation and progression through oocyte maturation. Although the purpose of numerous proteins' translation during oocyte maturation has been elucidated, the specific function of other proteins has not.

2.2. Cytoplasmic maturation and maternal factors

There is a population of proteins that are translated during oocyte maturation, yet their translation does not seem to be required for meiotic maturation. Rather, they might be involved in processes that occur after the metaphase II arrest, such as fertilization. Other cellular events that occur during oocyte maturation, such as organelle redistribution, also demonstrate the same behavior: they are needed only after the metaphase II arrest. Specific examples of protein translation and other cellular events are discussed below. While events leading to the segregation of homologous chromosomes (meiosis I) and subsequent arrest (in meiosis II) during oocyte maturation is often termed "nuclear maturation", protein accumulation and organelle redistribution not required for meiotic maturation can be termed "cytoplasmic maturation". Cytoplasmic maturation is critical for proper embryo development and is used to score oocyte "health" in IVF treatments (Combelles et al., 2002; Watson 2007). In addition to protein stockpiling and organelle redistribution, RNA accumulation has been shown to be important for events after the metaphase II arrest. Although RNA is accumulated before oocyte maturation, during oogenesis, and is thus not explicitly considered part of cytoplasmic maturation, these molecules remain an important part of embryogenesis. These three elements (RNA and protein accumulation, and organelle redistribution) demonstrate how events occurring in parallel to nuclear maturation are dispensable for this maturation, but important for events afterwards. In the interest of simplicity, organelle redistribution will be discussed under "cytoplasmic maturation", while RNA and proteins will be discussed under "maternal factors".

2.2.1. Cytoplasmic maturation

Many morphological events of oocyte maturation prepare the oocyte for fertilization and embryo development (Terasaki et al., 2001; Ferreira et al., 2008). The movement of cortical granules, for one, is used as a marker of oocyte health in IVF treatments. Cortical granules (CG) are small vesicles that are critical to the prevention of polyspermy (the fertilization of one egg by multiple sperm). During oocyte maturation, CGs grow in numbers and concentrate near the cortex of the plasma membrane (Cran and Cheng, 1985). Upon fertilization, they exocytose and expulse their contents into the perivitelline space. This is the area between the oocyte's plasma membrane and either the vitelline membrane (in *Xenopus* and other amphibians) or the zona pellucida (in mammals) that surrounds it. This process leads to a block of polyspermy (Ducibella et al., 1990; Schroeder et al. 1990).

Another example of organelle redistribution is that of the endoplasmic reticulum (ER). At fertilization, one of the first signaling cascades involves a calcium wave. These calcium stores come from the ER. Proper ER organization is necessary for the propagation of this calcium wave (Terasaki et al., 2001). Modifications to cortical ER has also been shown to be important for CG exocytosis (Campanella et al., 1984).

It has not been tested, to the best of my knowledge, whether or not CG movements and ER redistribution are necessary for nuclear maturation. However, their role in events after metaphase II arrest (i.e. fertilization and block to polyspermy) would suggest they are not required for nuclear maturation.

2.2.2. Maternal factors

Specific RNA and protein accumulated prior to fertilization form an important part of early embryo development. RNA transcription is not detected during the initial stages of *Xenopus* embryogenesis (Newport and Kirschner, 1982). The RNA stockpiled during oogenesis is first involved in the ZGA (Minami et al., 2007). Maternal RNA is also important in the establishment of the axes (ventral-dorsal, anterior-posterior, left-right) of the developing embryo (Heasman 2006). By creating a gradient or localization of specific RNA inside the oocyte, these RNA can direct cell fates. An example of this is VegT mRNA. VegT protein is a transcription factor that induces formation of specific cell lineages during embryo development. VegT mRNA is accumulated during oogenesis and localized to the vegetal half of the oocyte (Zhang and King, 1996). Thus, only cells that develop from the vegetal section of the oocyte will inherit VegT RNA, directing cell fates in a specific region of the embryo.

Regarding proteins, the role of glutathione (GSH) in fertilization and embryogenesis has been relatively well characterized (Eppig 1993 and Ferreira et al., 2008). Levels of GSH, a tripeptide, increase during oocyte maturation (Perreault et al., 1988). GSH is involved in sperm nucleus decondensation and male pronucleus formation (Yoshida et al., 1993; Sutovsky and Schatten, 1997). GSH is also an anti-oxidant, and was shown to be important in protecting the embryo from its high levels of reactive oxygen species (ROS) (You et al., 2010; Choe et al., 2010). Importantly, inhibition of GSH production during oocyte maturation resulted in fertilization defects (Calvin et al., 1986).

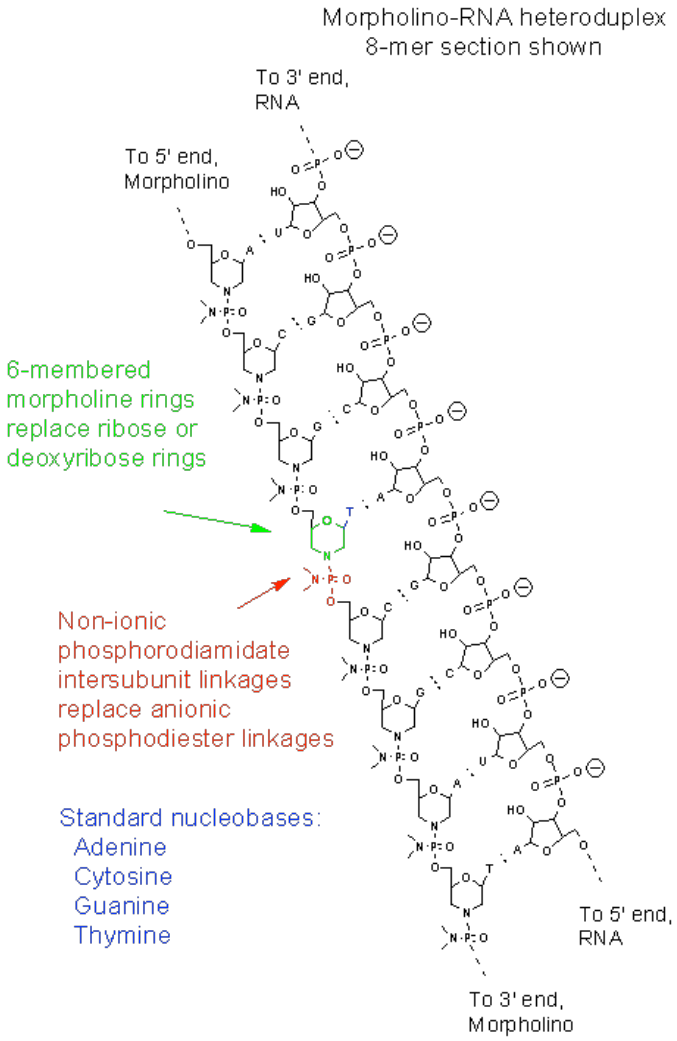
Examples of cytoplasmic maturation and maternal factors show how events occurring during oocyte growth and maturation (e.g. organelle redistribution or protein accumulation) are only essential much later in development, such as during embryogenesis.

3. The study of maternal factors in Xenopus laevis by means of the host transfer technique

In order to study the role of maternal factors, one must first modify the conditions of the target maternal factor. In the case of RNA and proteins, antisense oligos can be used to degrade RNA and/or inhibit their translation during oocyte maturation.

Injection of antisense (AS) oligonucleotides has been used to analyze both RNA and protein. AS oligos are used to deplete the oocyte of maternal RNA stores (Zhang et al., 1998). AS oligos bind to target RNA, which both inhibits their translation and triggers their degradation. However, there are certain downfalls to AS oligos that trigger degradation of the target RNA. These oligos have low sequence specificity and adverse non-specific effects that lead to similar phenotypes in both control and test groups (Summerton, 1999). To this end, morpholino oligonucleotides (morpholinos) can be used instead. Morpholinos bind to, and inhibit RNA activity or function, without resulting in the degradation of the target RNA molecule (Kloc et al., 2005). Morpholinos contain typical nucleic acid bases, but the backbone is composed of 6 member morpholine rings (rather than 5 member deoxyribose rings) linked by phosphorodiamidate groups (rather than phosphate groups) (Figure 2). The modifications in the structure of the morpholinos render them resistant to targeted degradation. After inhibition of translation with the use of morpholinos, the modified oocytes can be fertilized to observe their developmental competency.

Figure 2: Structure of a morpholino oligonucleotide. Morpholine rings linked by phosphorodiamidate groups form the backbone for typical nucleic bases. Image source: http://en.wikipedia.org/wiki/File:MorpholinoHeteroduplex.png.


Morpholino-RNA heteroduplex
8-mer section shown
To 3' end,
RNA
To 5' end,
Morpholino
6-membered
morpholine rings
replace ribose or
deoxyribose rings
Non-ionic
phosphorodiamidate
intersubunit linkages
replace anionic
phosphodiester linkages
Standard nucleobases:
Adenine
Cytosine
Guanine
Thymine
To 5' end,
RNA
To 3' end,
Morpholino

In the frog, eggs cannot be fertilized without the gelatinous membrane termed "jelly coat" (Yurewicz et al., 1975). The jelly coat is acquired as eggs pass through the oviduct of the frog after ovulation. Components of the jelly coat are required for sperm-egg binding and entry of the sperm into the egg. In order for *in vitro* matured oocytes (now called eggs) to acquire the jelly coat, donor eggs must be surgically transferred into a host frog. This process is referred to as the "host transfer technique".

The host transfer technique was developed by Heasman et al., (1991) in order to assess the role and function of maternally inherited mRNA. During ovulation in *Xenopus laevis*, the eggs are expulsed directly into the abdominal cavity, before being moved by ciliary action into the oviduct (Waring et al., 1941). This physiological particularity allows us to surgically transfer *in vitro* matured oocytes from a donor frog into the abdominal cavity of a host frog, after which these eggs pass through the oviduct and acquire the jelly coat. Once these eggs are deposited, they can be fertilized using isolated testes. Using the host transfer technique we can assess the impact of maternal factors on development.

4. Fertilization and embryogenesis in Xenopus laevis

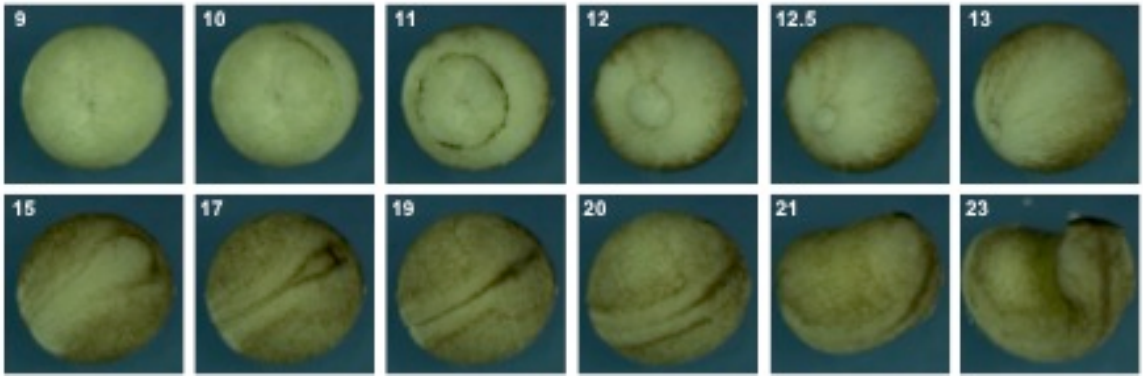
Sperm are first attracted to the egg by chemoattractants in the jelly coat. One identified chemoattractant, allurin, is a protein that binds to the surface of sperm and encourages motility of the sperm towards the egg (Burnett et al., 2008). The sperm then swims through the jelly coat and binds to receptors on the plasma membrane (Reinhart et al., 1998, Sakakibara et al., 2005). Binding triggers fusion of the sperm to the egg, cortical granule exocytosis (implicated in the block to polyspermy) and incorporation of the sperm nucleus

into the oocyte (Picheral and Charbonneau, 1982). Both the male and female nuclei, termed "pronuclei" then fuse and embryogenesis begins.

In *Xenopus laevis*, embryo development is scored from the 1 cell embryo (stage 1) to a tadpole (stage 50) (Nieuwkoop and Faber, 1994). The numbered stages do not refer to the number of cell divisions, but rather to key morphological events. After stage 50, the tadpole begins to form front and hind limbs, developing into a froglet, or small frog. The froglet will quickly lose its tail and then slowly grow over a period of about 6 months to a full-grown adult frog.

The first stage of embryogenesis is the cleavage stage. During this stage the embryo is simply a mass of undifferentiated, quickly dividing cells. In *Xenopus,* this lasts from stage 1 (1-cell embryo) to stage 4 (8-cell). Further division occurs during morulation (stage 5 to stage 7). Internal cell compaction during the next stage, called blastulation (embryo stages 8-9), results in the formation of a cavity inside the embryo, called the blastocoel. In the gastrula embryo (stages 10-12; Figure 3), the outer layers of cells begin to migrate to the vegetal pole and invaginate into the embryo to form a new cavity, called the archenteron. After gastrulation, the embryo begins organogenesis, where the various organs of the frog are formed. The first tissue to form is the neural tissue, in a process called neurulation (stages 13-19; Figure 3). In the neurula stage, the neural tube is formed. In stages 13-15, neural tissue on the dorsal surface of the embryo begins to thicken and form the neural crests. At stage 16, the neural crests begin to close and by stage 19, the neural tube is completely fused. The neural tube will eventually form parts of the central nervous system. Stages 20 through

Figure 3: Gastrulation and neurulation of *Xenopus laevis* embryogenesis. Number in upper left side of each image identifies the Nieukoop and Faber stage. Images taken from Xenbase.


9
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12.5
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17
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20
21
23

34 comprise the tailbud stages, where the embryo elongates and the rest of the major organs, as well as the tail, form. The tadpole further develops in stages 35-50. The most notable change during these final stages is a drastic increase in size.

Xenopus laevis development is interesting in the fact that the rate of development can be controlled by temperature (Nieuwkoop and Faber, 1994) At 23°C the first cleavage of the embryo occurs at 1.5 hours post-fertilization (hpf). The 11 subsequent divisions (up to stage 9) occur every 30min and are highly synchronous between blastomeres (individual cells of the early embryo), within ±2min of each other (Newport and Kirschner, 1982). However, these 30min divisions can be extended to 1h or more each if the embryos are cultured at temperatures as low as 13°C. At stage 10, after the 12th division, cellular division slows and becomes asynchronous within the embryo. This is also the timing of the ZGA in *Xenopus laevis* (Newport and Kirschner, 1982). If the embryo is maintained at 23°C from fertilization, it takes approximately 10, 15, 22 and 50 hours to reach the gastrula, neurula, tailbud and tadpole stage, respectively (Nieuwkoop and Faber, 1994).

5. Mitotic proteins as maternal factors

Our goal is to locate more proteins displaying maternal factor properties. More specifically, we wish to identify proteins that are accumulated during oocyte maturation but whose translation is dispensable for nuclear maturation. To further define our search, we examined the developmental needs of the early *Xenopus* embryo, and which specific proteins might be required in abundance during embryogenesis.

As stated above, the *Xenopus laevis* ZGA occurs after 12 rounds of cell division. Because of the high demand on mitotic proteins during this time, we have explored these proteins in hopes of identifying novel maternal factors. Although proteins can still be translated during embryogenesis, mitosis is rapid in the early *Xenopus* embryo. It is possible there is no time to translate all the proteins necessary for division of the large embryo. In fact, the translation of three mitotic proteins is upregulated during oocyte maturation: Aurora A (Aur-A; Ma et al., 2003), regulator of chromosome condensation 1 (RCC1; Dumont et al., 2007) and inner centromere protein (INCENP; Yamamoto et al., 2008). Aur-A is a kinase associated with the centrosomes and is implicated in microtubule elongation (Ducat and Zheng, 2004). RCC1 is involved in microtubule polymerization (Zheng, 2004). INCENP has roles throughout mitosis, primarily in chromosome segregation and cytokinesis (Eckley et al., 1997). Interestingly, Aur-A was originally discovered as Eg2, in a screen for mRNA whose translation is differentially regulated before and after fertilization (Le Guellec et al., 1991). The nine RNA identified in this search (labeled Eg1-9 in reference to their translation in eggs) are translated in the egg, but not after fertilization. Several of these RNA code for proteins that are involved in mitosis and cell cycle regulation (Blot et al., 2002), thus providing additional maternal factor candidates.

The inhibition of translation of RCC1 and INCENP during oocyte maturation does not appear to affect the progression of meiosis. Chromosomes in these modified oocytes arrest properly (Dumont et al., 2007) and protein levels of cell cycle markers, such as cyclin B, appear normal (Yamamoto et al., 2008). RCC1 and INCENP thus satisfy both of our requirements for maternal factors: they are actively translated during oocyte maturation but

appear dispensable for nuclear maturation. We decided to focus our initial analyses on INCENP.

6. INCENP

INCENP has two known isoforms in *Xenopus laevis*, INCENPa and INCENPb, and one in humans (Bloom, 1993). It is a subunit of the chromosomal passenger complex (CPC) (Reviewed in Ruchaud et al., 2007). This complex is composed of aurora-B, INCENP, survivin and borealin. While survivin and borealin act as scaffolding proteins, INCENP regulates aurora-B, which is the kinase of the CPC. The CPC is termed as such because all subunits follow the chromosomes during mitosis. They are located across the arms of the chromosomes in prophase. At metaphase, the CPC localizes to the centromeres. After anaphase, the CPC translocates to the central spindle, an area of the microtubule spindle that is thought to direct cytokinesis. In telophase, members of the CPC can also be found at the presumptive furrow, where the cytokinetic contractile ring will form. During the final stages of cytokinesis, the CPC is localized to the midbody, the bundle of the microtubules that is found between daughter cells.

Mutational analyses and knock-outs of various components of the CPC have shown that this complex is critical for proper chromosomal segregation, as well as cytokinesis. The CPC is critical for proper bi-polar spindle attachment to the kinetochores of chromosomes. During metaphase, microtubules coming from the centrosomes attach to protein complexes, called kinetochores, located in specialized regions of each chromosome. Proper and equal segregation of chromosomes during anaphase requires that equal numbers of microtubules

(coming from opposite centrosomes) attach to each kintetochore of each sister chromatid of a chromosome. The CPC is implicated in detecting and detaching unequal and unstable microtubule-kinetochore attachments (Kotwaliwale and Biggins, 2006). As evidence, a dominant mutant form of INCENP results in misalignment of chromosomes in mitosis, which leads to defects in cytokinesis (Mackay et al., 1998). Also, INCENP-null mice are embryonic lethal, resulting from severe chromosome segregation defects (Cutts et al., 1999).

INCENP might also be involved in cytokinesis, probably through functions of the CPC. After chromosomes have segregated during anaphase, a section of the plasma membrane begins to invaginate. This is the furrow, which is formed by constriction of the contractile ring, an assembly of proteins that "constrict" the plasma membrane during cytokinesis. Just before chromosome segregation, INCENP localizes to the presumptive furrow, the region of the plasma membrane that will become the furrow (Earnshaw and Cooke, 1991). Inhibition of the translocation of INCENP to the presumptive furrow (by injection of a dominant mutant form of INCENP) prevents the completion of cytokinesis (Eckley et al., 1997).

Hypothesis

Considering the increase of INCENP levels during oocyte maturation, the rapid mitosis that occurs during early embryogenesis, and the protein's importance in mitosis, it is considered a prime candidate as a maternal factor. Using the host transfer technique, we tested the hypothesis that the INCENP accumulated during oocyte maturation is a maternal factor required for *Xenopus laevis* embryo development.

Materials and Methods

Buffer List (taken from Sive et al. 2000)

Oocyte culture medium (OCM)

3:2 vol. ratio of L-15 prepared medium (Invitrogen, containing L-glutamine, cat# L4386) to ddH_2O

0.04% Bovine Serum Albumin (BSA)

5ml/L Gentamycin

1X Modified Barth's Solution (MBS)

7mL 0.1M $CaCl_2$

100mL 10X MBS

893mL ddH_2O

10X MBS

880mM NaCl

10mM KCl

10mM $MgSO_4$

50mM HEPES

25mM $NaHCO_3$

pH to 7.5

Phosphate Buffered Saline (PBS) Buffer

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

ddH_2O to 1L

pH to 7.4

Oocyte Ringer 2 plus calcium (OR2++)

1:1:8 volume ratio of Stock A: Stock B: ddH_2O

pH to 7.8

add 2mL 0.5M $CaCl_2$

1mL 10mg/mL gentamycin

Stock A

48.221g NaCl

1.864g KCl

2.03g $MgCl_2$-H_2O

11.915g HEPES

1.52g NaOH

ddH_2O to 1L

Stock B

1.42g Na_2HPO_4

ddH_2O to 1L

Lysis Buffer

1000X dilution of lysine

1000X dilution of protease inhibitor cocktail

100X okadaic acid

in EB buffer

EB buffer

20mM HEPES pH 7.3

80mM glycerolphosphate pH 7.3

20mM EGTA

15mM $MgCl_2$

1mM DTT

2X Sample Buffer

5mL 1M Tris pH 6.8

4.6mL ddH_2O

8mL glycerol

16.4mL 10% SDS

2.0mL 0.1% Bromophenol blue

add 10% beta-mercaptoethanol before use

Blocking solution

5% powdered fat-free milk in TBST

TBST

120mL 1M Tris pH 7.5

360mL 5M NaCl

2.4mL Tween 20

11.52L ddH_2O

Primary antibody solution

500X dilution of INCENP antibody (Cat# ab12187, Abcam, USA) in blocking solution

Secondary antibody solution

3mL of ECL™ Anti-rabbit IgG Horseradish peroxidase linked whole antibody (from Donkey) (cat# NA934V, GE Healthcare, UK) in 10mL Blocking solution

MEMFA fixation buffer

0.1M MOPS pH 7.4

2mM EGTA

1mM $MgSO_4$

3.7% formaldehyde

Oocyte culture and injection

Adult, oocyte positive female *Xenopus laevis* frogs were obtained from Nasco, USA. They were primed using pregnant mare serum gonadotropin (PMSG, cat# G4877, Sigma, USA) to stimulate oogenesis, thus increasing the number of fully-grown stage VI oocytes. To prime, frogs were injected with 100UI of PMSG into the dorsal lymph node 3-5 days before collecting oocytes. The frogs were then sacrificed by decapitation and oocytes were isolated from the ovaries by manual defolliculation. The oocytes are found in between layers of follicle cells that compose the membrane of the ovaries. Using forceps, one can manually remove the layers of follicle cells (called "defolliculation") that surround the oocyte, isolating it from the ovary. Defolliculated oocytes were then stored in oocyte culture medium (OCM) at 18°C.

To inhibit translation of INCENP, oocytes were injected with a morpholino oligonucleotide anti-sense to the region surrounding the start codon of *Xenopus* INCENP mRNA ($INCENP_{morpho}$; 5'GGGACAGGCACTCTGCATCGTTCAT3'; Gene Tools, USA). The control morpholino ($ctrl_{morpho}$) contains the same nucleotides but in a scrambled sequence (5'GGAGCAGGAGACGCCTCACTTTTCT3') provided by Gene Tools. GV oocytes were injected with 10nl of a 1mM concentration solution of morpholinos into the equatorial region using a fine needle and PLI-100 PICO-INJECTOR (Warner Instruments, USA) gas pressure injection system.

Oocytes were matured in OCM containing 1uM of progesterone (Sigma, USA) and incubated overnight at 18°C.

Creation of INCENP rescue constructs

The sequences of INCENPa (Reference Number: NM_001088421) and INCENPb (Reference Number: NM_001137571) were obtained from the National Centre for Biotechnology Information's (NCBI) Nucleotide database on March 17th, 2010. These sequences were used to probe for clones in the National Institutes for Basic Biology's (NIBB) *Xenopus* Database (XDB) v3.2 on March 17th, 2010. Two clones were returned, corresponding to INCENPa (XDB clone: XL157o01) and INCENPb (XDB clone: XL018p07). Clone inserts were excised from their respective plasmids by PCR using primers against INCENPa (forward: 5'-CCGGAATTCAACGATGCAGAGTGCCT-3'; reverse: 5'-CCGGAATTCGTATTTGAGGCCATAACCC-3') and INCENPb (forward: 5'-

CCGGAATTCAACGATGCAGAGTGCCT-3'; reverse: 5'-CCGGAATTCATATTTGAGGCCGTAACCC-3'). These inserts were then ligated into PCS2+HA vectors (Booth et al., 2002), creating HA-INCENPa and HA-INCENPb.

Preparation and injection of mRNA

For fluorescent microscopy, GV oocytes were injected with mRNA coding for proteins tagged with fluorescent peptides. For visualization of chromosomes, mRNA coding for histone H2B, tagged with a red fluorescent protein (mRFP) peptide, was used (A gift from Dr William M. Bement, University of Wisconsin, USA). Histone H2B is a component of chromatin and allows us to follow chromosomes. To visualize the plasma membrane, mRNA coding for a domain of utrophin, tagged with a green fluorescent protein (GFP), was used (A gift from Dr William M. Bement, University of Wisconsin, USA). The calponin homology domain of utrophin binds to polymerized F-actin (Man et al., 1992), and allows us to visualize F-actin at the plasma membrane cortex (Burkel et al., 2007). 5-10nl of a 500-1000ug/ml solution of mRNA was injected into GV oocytes, in the same manner as, and along with, the morpholino solutions (described above).

The DNA sequences coding for all mRNAs used were contained in PCS2+ plasmid vectors (Booth et al., 2002). The constructs were linearized with *Not*I and mRNA was transcribed using the mMessage mMachine *in vitro* transcription kit (cat# AM1340, Ambion, USA). mRNA was then quantified using a Qubit™ Fluorometer (cat# 32852, Invitrogen, USA).

Host transfer technique

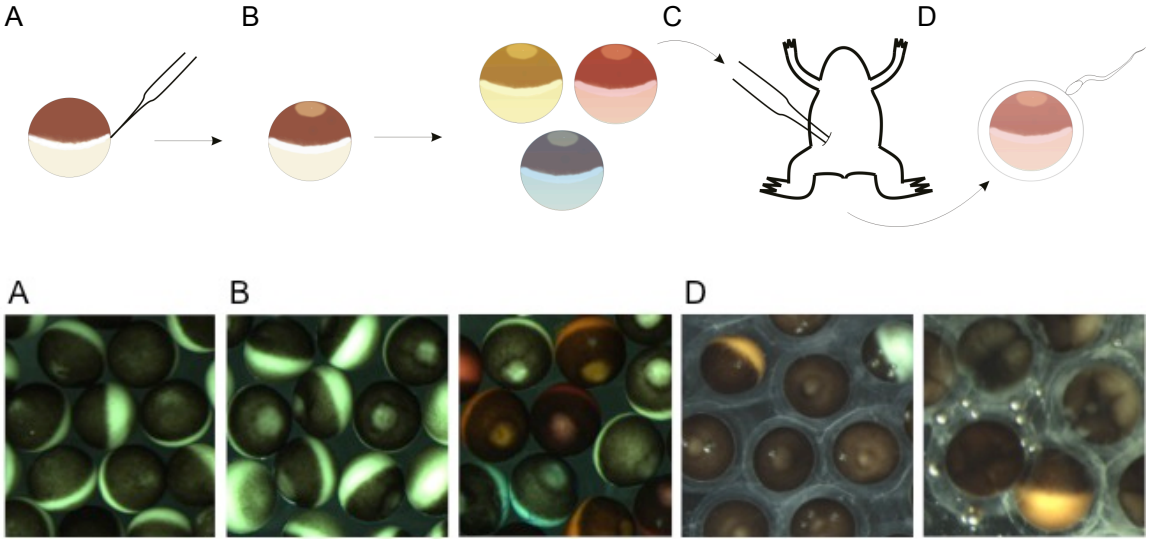
The host transfer technique (Figure 4) was performed as described by Heasman et al. (1991). Mature oocytes (eggs) were dyed using vital dyes (Sigma, USA) to identify $ctrl_{morpho}$ vs $INCENP_{morpho}$ groups. Eggs were then surgically transferred into the abdominal cavity of an ovulating female anesthetized with MS222 (Ethyl 3-aminobenzoate methanesulfate salt; cat# A5040, Sigma, USA). Ovulated eggs were fertilized *in vitro* with testes isolated from male *Xenopus laevis* frogs (also obtained from Nasco). Embryos were then dejellied at the 1 or 2 cell stage using a 2% cysteine solution (in H_2O, pH 8.0) for 2min. Embryos were cultured in 0.1X modified Barth's solution (MBS) at room temperature.

Embryo injections and manipulations

Embryos analyzed during early embryogenesis (Figures 10, 11) were injected with 42pg of morpholinos at the 1-cell stage in 3% Ficoll. Embryos analyzed during later stages of embryogenesis (Figure 12) were injected at the 2-cell stage with 21pg per blastomere of $INCENP_{morpho}$ or standard control (SC_{morpho}; 5'-CCTCCTACCTCAGTTACAATTTATA-3'). Embryos were cultured in 0.1X MBS.

$INCENP_{morpho}$ embryos that were analyzed for presence of multiple nuclei were fixed in a 2% paraformaldehyde (in PBS buffer) for 2 hours and washed twice in PBS buffer for 1 hour/wash.

Figure 4: Host transfer protocol (Adapted from Heasman et al. 1991). Upper panel series shows a flowchart of the protocol. Lower panels shows images of live oocytes and embryos. Morpholino oligonucleotides antisense to the mRNA of mitotic proteins were used to inhibit translation of specific proteins during oocyte maturation. **A,** Morpholinos are injected into immature oocytes. **B,** The oocytes are matured in progesterone and the different test groups are dyed using vital dyes. **C,** Oocytes are then surgically transferred into an ovulating female frog. **D,** Eggs are deposited, acquiring the jelly coat necessary for subsequent fertilization.


A
B
C
D
A
B
D

Confocal fluorescence microscopy

Oocytes were analyzed for defects in meiosis. Imaging was performed using a 60X oil objective on a Zeiss Axiovert microscope with a BioRad 1024 laser scanning confocal imaging system. Fixed embryos were analyzed for defects in cytokinesis and/or mitosis using a 20X objective. Images were rendered to 3D and analyzed using Volocity imaging software (PerkinElmer, USA).

Parthenogenetic activation of GVBD oocytes

Mature, metaphase II arrested oocytes were stimulated to emit the second polar body by pricking them in the animal hemisphere with a fine needle (needle tip diameter: approximately 10um) in OR2++ buffer (Kemp, 1961; Kemp and Istock, 1962). Pricked oocytes are placed upside down on a confocal microscope (see above). Oocytes are scanned for the duration of second polar body emission. Sister chromatid separation occurs approximately 12 minutes after pricking and completion of second polar body emission takes an additional 15 minutes.

Western blotting

Western blotting was performed as described in Ma et al. (2003). 10 oocytes or five embryos were lysed per group. Oocytes/embryos were placed in a clean tube and all

medium was removed. Oocytes/embryos were then lysed by addition of Lysis Buffer and repeated pipetting to crush the oocytes, creating a lysate. 5ul of Lysis Buffer was used per oocyte/embryo. This lysate was then centrifuged for 30min at 15000g in a J-251 Avanti™ Centrifuge (Beckman Coulter, USA). This creates a three-layered solution, with a cell debris pellet, an aqueous protein solution in the middle and a lipid layer surfactant. The protein solution was retrieved and an equal volume of SDS Sample Buffer was added to the protein solution. These protein samples were then stored at -20°C. Protein samples were heated at 85°C for 10min and run on a 10% polyacrylamide gel. The equivalent of one oocyte/embryo was loaded per lane and samples were run at 120V for approximately two hours. The gel was then washed in Transfer buffer for 10min. Proteins were transferred to a nitrocellulose membrane for one hour at 9V. The nitrocellulose membrane containing transferred protein samples was incubated for one hour in a Blocking solution, and then incubated overnight in the Primary antibody solution. The primary antibody used was a polyclonal antibody raised in rabbit and targets amino acids 884-901 of human INCENP (Cat# ab12187, Abcam, USA). This antibody also recognizes *Xenopus laevis* INCENP (Andersen et al., 2008). The nitrocellulose membrane was washed 3 times for 10 minutes in TBST, and then incubated for one hour in the Secondary antibody solution. The secondary antibody used was ECL™ Anti-rabbit IgG conjugated to horseradish peroxidase, from donkey (GE Healthcare, UK). Proteins were visualized on film by chemiluminescence using ECL™ Western Blotting Detection Reagents (cat# RPN2106, Amersham Biosciences, USA).

whole mount in situ *hybridization*

Whole mount *in situ* hybridization (WISH) was performed as described by Harland (1991) with modifications described in Goda et al. (2009). WISH is comparable in principle to Western and Northern blots. Antisense-RNA probes are used to detect their respective mRNA target in whole embryos. The probes are transcribed using the same methods as described above for mRNA, with addition of a DIG labeling mix (Roche, Germany) which incorporates digoxygenin(DIG)-labeled nucleotides into the RNA probe. This DIG tag can be detected with an anti-DIG antibody (Roche, Germany) and visualized with BM purple (Roche, Germany). Negative control probes consist of the sense-RNA corresponding to each antisense-RNA probe.

Embryos are first fixed at various stages in MEMFA. Embryos are then bleached using peroxide. Using an automated InsituPro (INTAVIS Bioanalytical Instruments, Germany) machine, a series of washes and incubations with the DIG-labeled probe and anti-DIG antibody are performed on the fixed embryos. The anti-DIG antibody is conjugated to alkaline phosphatase (AP). When BM purple (an alkaline phosphatase substrate) is added to the solution, the AP converts it to a dark purple precipitate, identifying the location of the target mRNA.

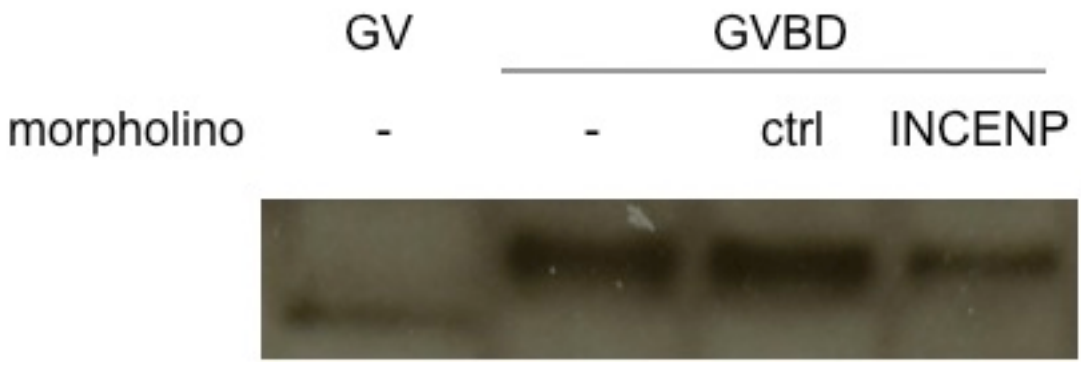
Results

Note: Creation of INCENP rescue constructs, as well as experiments summarized in figures 12 and 13 took place at the National Institute of Basic Biology in Okazaki, Japan. Experiments were performed by Geoffrey Leblond, with the help of Dr. Makoto Suzuki and Chiyo Takagi, under the supervision of Dr. Naoto Ueno.

Inhibition of INCENP during oocyte maturation

Our first step was to inhibit the increase in INCENP protein levels that occurs during oocyte maturation (Yamamoto et al., 2008). This was done using morpholino oligonucleotides anti-sense to a region surrounding the start codon of the INCENP mRNA (Yamamoto et al., 2008). GV oocytes contain a level of INCENP stockpiled during oogenesis, which will be referred to as "stockpiled INCENP" (Figure 5, lane I). Addition of progesterone to the oocyte culture medium triggers GVBD and progression of the oocyte through oocyte maturation. GVBD also correlates with the appearance of a white spot at the animal pole (Figure 4). INCENP is accumulated during oocyte maturation (Figure 5, lane 2). This GVBD level of INCENP will be referred to as "accumulated INCENP". Also noticeable in the GVBD sample, is the shift to an apparently higher molecular weight after oocyte maturation, indicative of INCENP's phosphorylation by Aur-B during oocyte maturation (Yamamoto et al., 2008). Two morpholinos were used in this study. The INCENP morpholino ($INCENP_{morpho}$), as described above, and a control morpholino ($ctrl_{morpho}$), which contains the same nucleotides as $INCENP_{morpho}$, but in a scrambled sequence. To inhibit translation, morpholinos were injected at the GV stage. Injection of $ctrl_{morpho}$ (lane 3) did not

Figure 5: Stockpiled level of INCENP remains after morpholino injections. INCENP protein levels before (GV) and after (GVBD) oocyte maturation, with and without injections of $ctrl_{morpho}$ or $INCENP_{morpho}$.


GV
GVBD
morpholino
-
-
ctrl
INCENP

affect the increase in INCENP protein nor its gel shift. Injection of $INCENP_{morpho}$ (lane 4) inhibited the increase in INCENP levels but did not affect the shift.

$INCENP_{morpho}$ effect on nuclear maturation

Part of our hypothesis entails that as a maternal factor, the accumulated INCENP protein would be dispensable for the transition from meiosis I to meiosis II that occurs during oocyte maturation (i.e. nuclear maturation). Yamamoto et al. (2008) demonstrated that inhibiting INCENP translation did not affect the normal fall and rise in the levels of cyclin B during oocyte maturation. To confirm that nuclear maturation was indeed not affected, we observed emission of the 1st polar body (PB) in $INCENP_{morpho}$ embryos. We used fluorescent probes to track chromosomes in the egg and in the polar body. Oocytes were stimulated to progress through oocyte maturation following injection with morpholinos and probes. Once arrested at metaphase II, the oocytes were scanned using a confocal microscope. Figure 6 shows that after injection of $ctrl_{morpho}$ or $INCENP_{morpho}$, chromosomes recongress and arrest into a proper metaphase II plate. In both groups, a PB can also be seen above the plasma membrane, confirming that 1st PB emission was successful. 10 out of 11 $ctrl_{morpho}$ oocytes emitted the 1st polar body. (Table 1) The one $ctrl_{morpho}$ oocyte that did not emit the 1st polar body had an abnormal spindle. 15 out of 15 $INCENP_{morpho}$ oocytes emitted the 1st polar body. These results are representative of two separate experiments.

Figure 6: INCENP$_{morpho}$ does not interfere with first polar body emission. Confocal microscopy of oocytes arrested in metaphase of meiosis I. Oocytes were injected with ctrl$_{morpho}$ or INCENP$_{morpho}$ and stimulated with progesterone overnight. Upper panels show the top view, looking down onto the plasma membrane. Lower panels show side view. Chromosomes are visualized using mChe-H2B (red). In the model between panels, the 1st polar body is shown above the plasma membrane, with the metaphase I spindle bellow. The location of the plasma membrane in the live scans is marked by a dashed line.

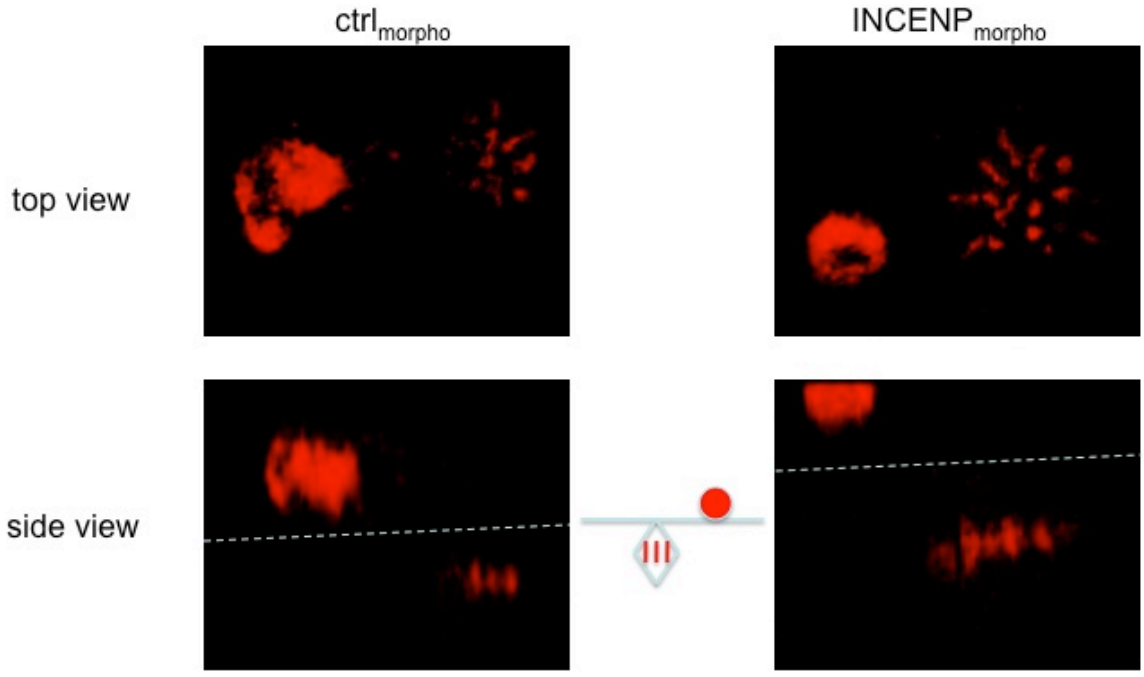

ctrl morpho
INCENP morpho
top view
side view

Table 1: Number of oocytes having emitted 1st and 2nd polar bodies after injection with morpholinos. Oocytes were injected with 10pmol of $ctrl_{morpho}$ or $INCENP_{morpho}$, along with 10ng of mChe-H2B to allow visualization of chromosomes. 1st polar body was scored after overnight stimulation with progesterone. 2nd polar body was scored after prick-activating mature eggs. [a] The single oocyte that did not appear to have emitted first polar body contained an abnormal spindle after hormone stimulation. [b] All oocytes not having emitted 2nd polar body did not activate after prick-activation.

	1st Polar body emitted	2nd Polar body emitted
$ctrl_{morpho}$	10/11[a]	4/6[b]
$INCENP_{morpho}$	15/15	4/7[b]

INCENP$_{morpho}$ effect on development

Since it appeared that INCENP$_{morpho}$ injections had no effect on nuclear maturation, we wished to test the developmental capacity of INCENP$_{morpho}$ injected oocytes. One of the first events of development is the resumption and completion of meiosis from its second arrest, including the emission of the 2nd PB. Oocytes arrested at metaphase II were pricked with a fine needle to mimic fertilization and release the oocyte from its arrest, stimulating 2nd PB emission. Figure 7 shows the progression of oocytes injected with either ctrl$_{morpho}$ or INCENP$_{morpho}$, from a metaphase II arrest (column 1), through the separation of the chromosomes at anaphase II (column 2), and finally after the emission of the 2nd PB (column 3). In both groups, the 2nd PB can be seen and is properly emitted, suggesting accumulated INCENP is not necessary for 2nd polar body emission. 4 out 6 ctrl$_{morpho}$ oocytes, and 4 out of 7 INCENP$_{morpho}$ oocytes emitted the second polar body. (Table 1) All oocytes that did not emit the 2nd polar body did not activate. These results are representative of two separate experiments.

The most important and convincing test of developmental capacity is the ability for a fertilized egg to develop as an embryo. To this end, mature oocytes (eggs) were subjected to the host transfer technique (Heasman et al., 1991). By inserting eggs into the abdominal cavity of a host frog, the eggs can be laid *in vivo*. During this process they acquire the jelly coat, which is required for fertilization (Yurewicz et al., 1975). After fertilization, the embryos were followed for any developmental defects. At stage 4, 82% of the ctrl$_{morpho}$ embryos (total n=45) were at the expected 8-cell stage and developing normally, whereas only 4% of INCENP$_{morpho}$ embryos (total n=56) were normal (Figure 8, "normal cleavage").

Figure 7: INCENP$_{morpho}$ does not interfere with second polar body emission.
Confocal microscopy time course of oocytes progressing through meiosis II. Oocytes were injected with ctrl$_{morpho}$ or INCENP$_{morpho}$. Oocytes were pricked with a sharp needle to simulate fertilization and to activate the oocytes. 1st and 2nd polar body (PB1 and PB2, respectively) are marked. Chromosomes are visualized using mChe-H2B (red). In the model of polar body emission shown between panels, the plasma membrane is marked by a blue line and the microtubule spindle is shown in green. The location of the plasma membrane in the live scans is marked by a dashed line. Scale bar = 8um.

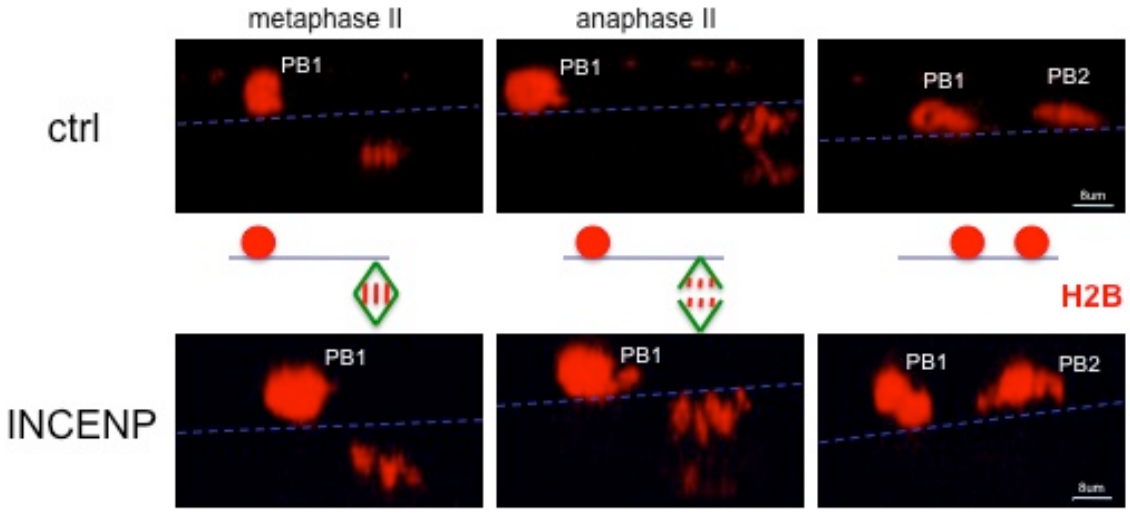
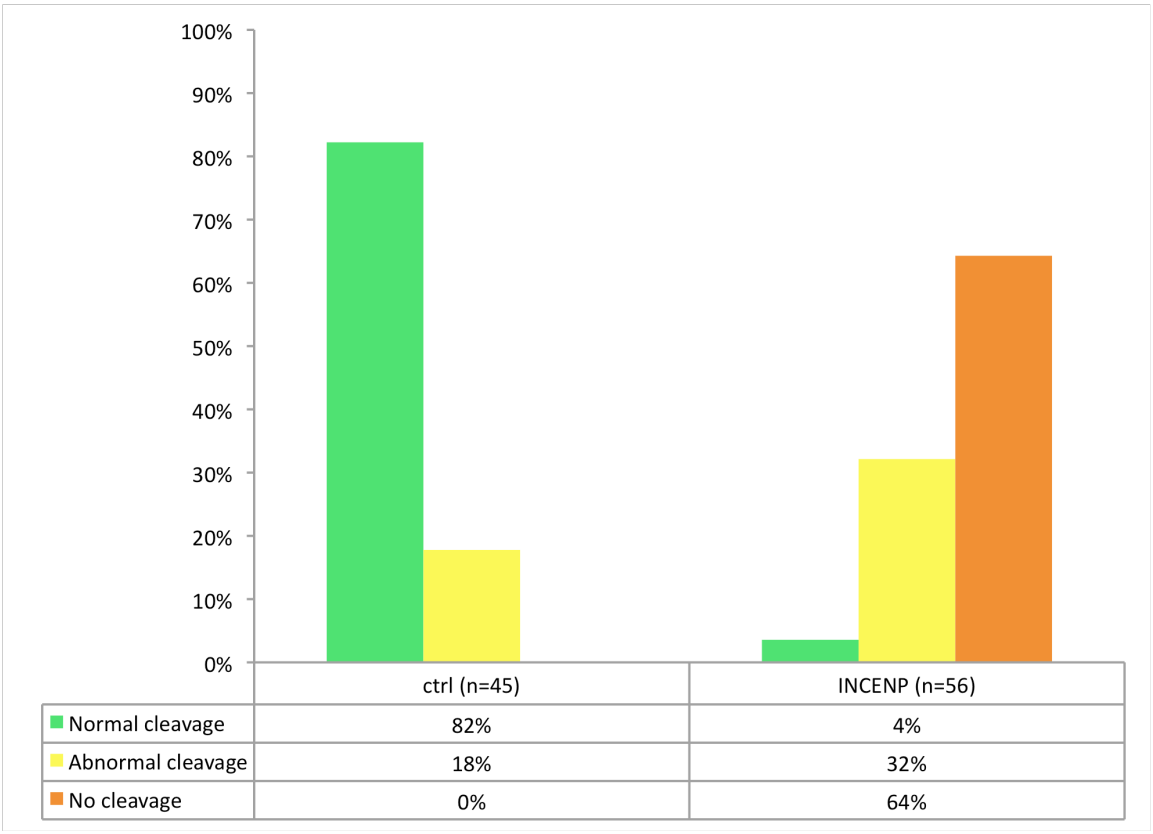
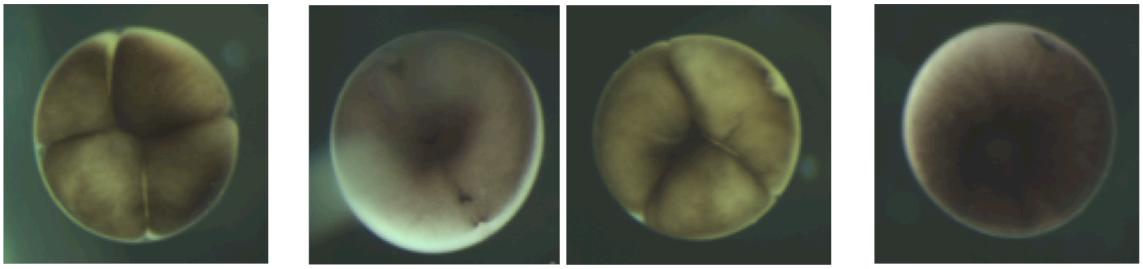

metaphase II
anaphase II
ctrl
PB1
PB1
PB1
PB2
8um
H2B
INCENP
PB1
PB1
PB1
PB2
8um

Figure 8: INCENP translation during oocyte maturation is essential for early embryogenesis. Embryos were analyzed at stage 4 (8-cell). Oocytes were injected with either $ctrl_{morpho}$ or $INCENP_{morpho}$, subjected to host transfer technique and fertilized. Embryos either cleaved normally (Normal cleavage), experienced abnormal or partial cleavage (Abnormal cleavage) or showed no signs of cleaving (No cleavage). *n* refers to the number of total embryos recovered per group.



	ctrl (n=45)	INCENP (n=56)
Normal cleavage	82%	4%
Abnormal cleavage	18%	32%
No cleavage	0%	64%



Normal cleavage Abnormal cleavage No cleavage

18% of $ctrl_{morpho}$ embryos and 32% of $INCENP_{morpho}$ embryos exhibited asymmetrical or partial cleavage patterns. Either the embryos contained an odd number of cells or displayed furrows (invaginations of the plasma membrane) that never completely cleaved the embryo (Figure 8, "abnormal cleavage"). 64% of $INCENP_{morpho}$ embryos exhibited a unique phenotype (Figure 8, "no cleavage"). These embryos (as did all other groups) showed physiological signs of activation, such as lifting of the vitelline membrane from the egg or contraction of the animal pigmentation. In figure 8, the image labeled "no cleavage" represents an $INCENP_{morpho}$ embryo (viewed from the top) in which the pigment on the surface of the cell is contracting towards the animal pole, or "top" of the embryo. This particular group of $INCENP_{morpho}$ embryos, however, showed no furrows and lacked any sign of cleavage.

Rescue of $INCENP_{morpho}$ phenotype

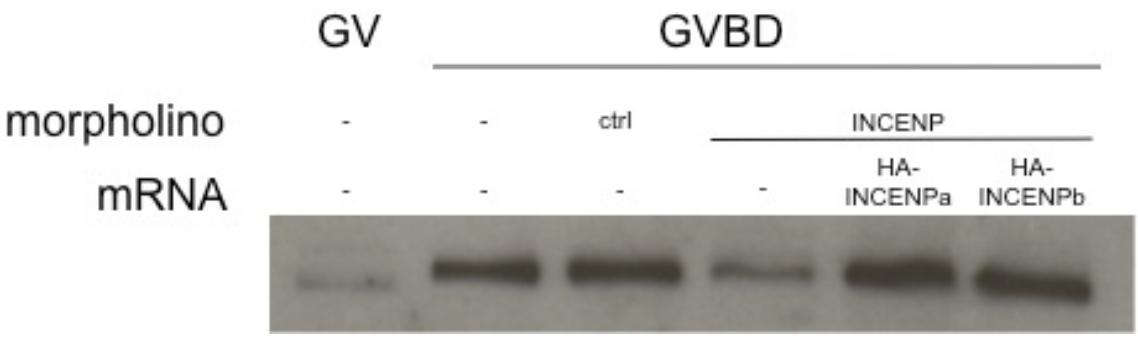
To further confirm that accumulated INCENP was specifically required for early embryogenesis, we performed rescue experiments in which INCENP mRNA was co-injected with $INCENP_{morpho}$. These results are described and discussed separately because they are based on only one experiment, whereas other experiments (unless otherwise stated) were repeated at least three times.

In Japan, I constructed 2 rescue plasmids, expressing either INCENPa or INCENPb isoforms (HA-INCENPa and HA-INCENPb, respectively). mRNA made *in vitro* from these plasmids were co-injected with $INCENP_{morpho}$ to rescue the effects of this morpholino. The rescue mRNAs are not affected by $INCENP_{morpho}$ because the INCENPa/b sequence is preceded by

a sequence coding for an HA-tag. The HA-tag is an epitope tag derived from haemagglutinin. Morpholinos only inhibit translation of a target mRNA when they target the 5' untranslated region preceding the ATG start codon or the area immediately following the ATG (Summerton 1999). $INCENP_{morpho}$ targets the ATG start codon of INCENPa/b mRNA, as well as 22 nucleotides following the ATG. By adding a 27 nucleotide sequence before the ATG start codon (thereby generating HA-INCENPa/b) we effectively push the target sequence of the $INCENP_{morpho}$ downstream by 27 nucleotides. This renders the morpholino ineffective at inhibiting translation of these mRNA. Co-injection of either HA-INCENPa or HA-INCENPb along with $INCENP_{morpho}$ (Figure 9, lanes 5 and 6, respectively) rescues the level of INCENP accumulated after oocyte maturation to levels similar to those in non-injected (Figure 9, lane 2) and $ctrl_{morpho}$ injected (Figure 9, lane 3) oocytes.

These rescue oocytes were also subjected to the host transfer technique in order to test their developmental competence. In the experiment performed, 4 groups of oocytes were injected with either $ctrl_{morpho}$ (number of recovered embryos from this group: n=4), $INCENP_{morpho}$ (n=12), $INCENP_{morpho}$ + HA-INCENPa (n=7), $INCENP_{morpho}$ + HA-INCENPb (n=1). All four (100%) of $ctrl_{morpho}$ embryos were developing normally at stage 4. Five (42%) of the 12 $INCENP_{morpho}$ embryos were cleaving abnormally at stage 4. Seven (58%) of the 12 $INCENP_{morpho}$ embryos exhibited no signs of cleaving at stage 4. Five (71%) of the seven HA-INCENPa embryos appeared to be developing normally at stage 4 while the other two (29%) were cleaving abnormally. The single (100%) INCENPb embryo recovered in the experiment was cleaving abnormally. These data suggest that HA-INCENPa/b can rescue the effects of $INCENP_{morpho}$.

Figure 9: HA-INCENPa and HA-INCENPb mRNA rescues accumulated INCENP protein levels. INCENP protein levels before (GV) and after (GVBD) oocyte maturation, with and without injections of $ctrl_{morpho}$ or $INCENP_{morpho}$. Two $INCENP_{morpho}$ groups were co-injected with either INCENPa or INCENPb mRNA.


GV
GVBD
morpholino
-
-
ctrl
INCENP
mRNA
-
-
-
-
HA-INCENPa
HA-INCENPb

INCENP translation during early embryogenesis

To confirm that the phenotype observed in Figure 8, where INCENP embryos do not cleave, is not due to inhibition of INCENP translation during early embryogenesis (rather than inhibition during oocyte maturation), morpholinos were injected directly into 1-cell embryos. 88% of $ctrl_{morpho}$ injected embryos (total n= 97) and 87% of $INCENP_{morpho}$ embryos (n=87) were cleaving normally at stage 4. (Figure 10) 88% of $ctrl_{morpho}$ injected embryos and 84% of $INCENP_{morpho}$ injected embryos were cleaving normally at stage 9.

Yamamoto et al.'s (2008) data suggested that INCENP might be degraded after fertilization. This was concluded from experiments using *Xenopus* egg extracts that were activated by addition of calcium, which mimics the calcium wave that occurs during fertilization. To examine this in the developing embryo, we analyzed protein samples of embryos injected at the 1-cell stage with $ctrl_{morpho}$ and $INCENP_{morpho}$, as well as from uninjected (host) embryos (Figure 11). A sample taken from unfertilized eggs (stage 0) from the same frog was used as a baseline level for accumulated INCENP (Figure 11, lane 1). Other protein samples were taken at stages 1, 2, 4 and 9 (Figure 11, lanes 2 through 9). In contrast to Yamamoto et al.'s study, levels of INCENP protein appeared to remain constant in all three groups in all time points sampled. Whole egg extracts were used in these experiments. Since calcium is known to trigger proteolysis in many other systems (Van Ginkel et al., 1993; Gulati et al., 2004; Wingertzahn and Ochs 1997), it is possible that in these *Xenopus* egg extracts, addition of calcium triggers global, non-specific calcium-mediated proteolysis.

Figure 10: Morpholinos injected after fertilization have no effect on early development. Embryos were injected with $ctrl_{morpho}$ or $INCENP_{morpho}$ at the 1-cell stage. The percentage of embryos cleaving normally at stages 4 and 9 are shown. *n* refers to the number of total embryos per group.

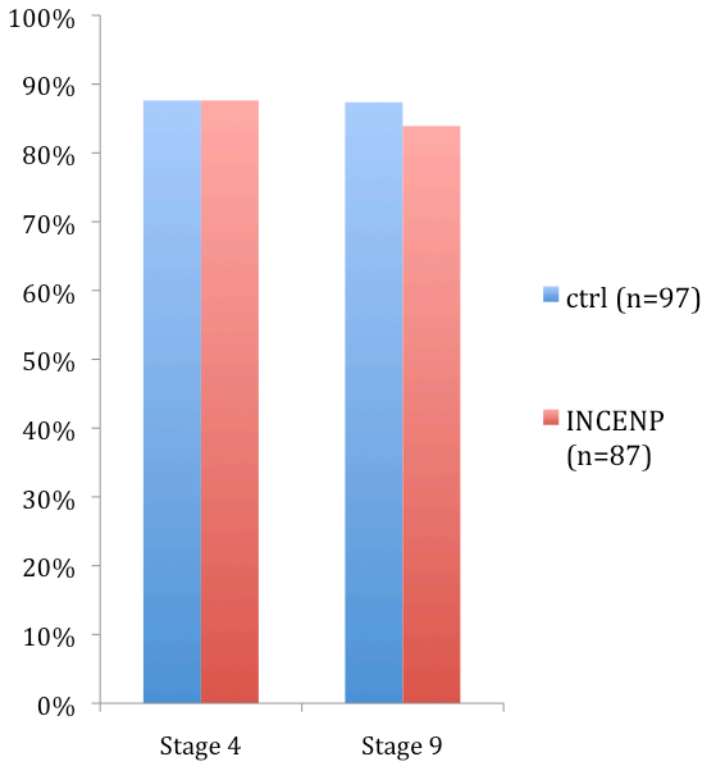
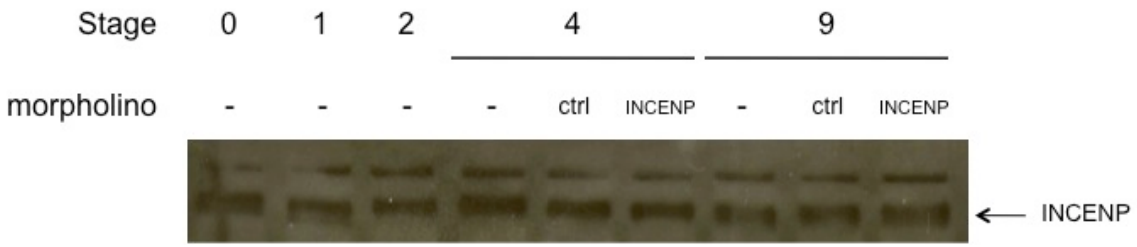

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Stage 4
Stage 9
ctrl (n=97)
INCENP (n=87)

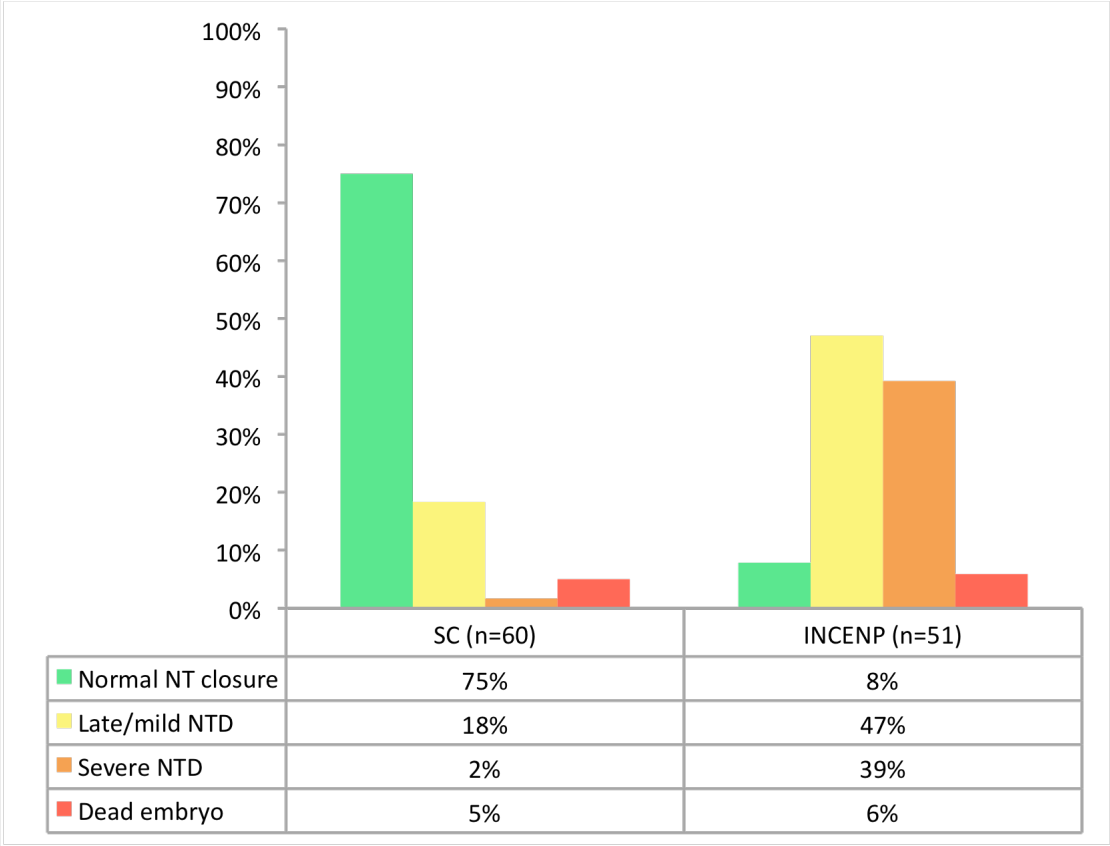
Figure 11: INCENP is not translated during early embryogenesis. Western blot showing INCENP protein levels during early embryogenesis, up to stage 9. Stage 0 refers to unfertilized eggs. Embryos were left uninjected, or injected at stage 1 with $ctrl_{morpho}$ or $INCENP_{morpho}$. The signal representing INCENP protein is shown.


Stage
0
1
2
4
9
morpholino
-
-
-
-
ctrl
INCENP
-
ctrl
INCENP
INCENP

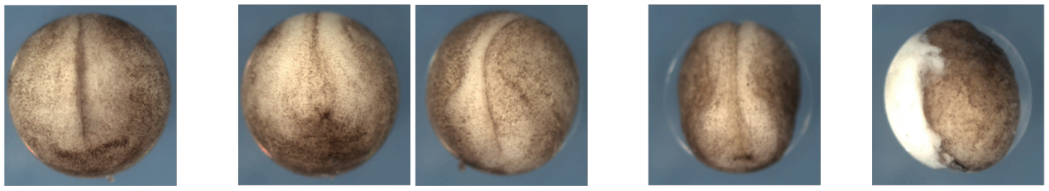
When injected in stage 1 embryos, $INCENP_{morpho}$ appears to have no effect on development or INCENP protein levels up until stage 9. We wanted to confirm that $INCENP_{morpho}$ could function in embryos, and inhibit INCENP translation. In Japan, I performed experiments in which injected embryos were followed later in development. Embryos were injected in both blastomeres at stage 2 (2-cell) with either $INCENP_{morpho}$ or a scrambled control morpholino (SC_{morpho}) and followed during development until a phenotype was observed. Both SC_{morpho} and $INCENP_{morpho}$ embryos developed normally through early embryogenesis (cleavage through blastulation), confirming our previous results. Both groups also progressed normally through gastrulation. However, $INCENP_{morpho}$ embryos showed defects in neural tube closure (NTC) during the neurulation, at stage 19 (Figure 12). Neural tube (NT) formation is a critical event in vertebrate embryogenesis (Patel et al., 2010). During NTC, neural tissue on the surface of the embryo rises and closes to form the NT that will eventually develop into the central nervous system. 75% of SC_{morpho} injected embryos (total n=60) completed NTC normally, whereas only 8% of $INCENP_{morpho}$ embryos (n=51) were normal (Figure 12). 18% of SC_{morpho} and 47% of $INCENP_{morpho}$ embryos exhibited late NTC or mild NT defects (NTD; where a portion of the neural tube remained unclosed). Strikingly, 2% of SC_{morpho} and 39% of $INCENP_{morpho}$ displayed severe NTD, in which case the entirety of the neural tube remained open. 5% of SC_{morpho} and 6% $INCENP_{morpho}$ exhibited cytoplasm in the perivitelline space, and were considered dead embryos. These results confirm that $INCENP_{morpho}$ can have an effect in embryos and validates the results described in figures 9 and 10.

To further characterize INCENP expression during embryogenesis, *in situ* hybridization of *Xenopus laevis* embryos was performed (Figure 13). mRNA probes were generated using

Figure 12: INCENP$_{morpho}$ causes defects in neural tube closure. Embryos were injected with standard control (SC$_{morpho}$) or INCENP$_{morpho}$. Percentages of embryos expressing various phenotypes at stage 19 are shown. "Normal NT closure" = fully closed neural tube (NT); "Late/mild NTD" = partially closed NT or mild NT defects (NTD); "severe NTD" = fully open NT; "Dead embryo" = embryos with cytoplasm in the perivitelline space. *n* refers to the number of total embryos per group.



	SC (n=60)	INCENP (n=51)
Normal NT closure	75%	8%
Late/mild NTD	18%	47%
Severe NTD	2%	39%
Dead embryo	5%	6%



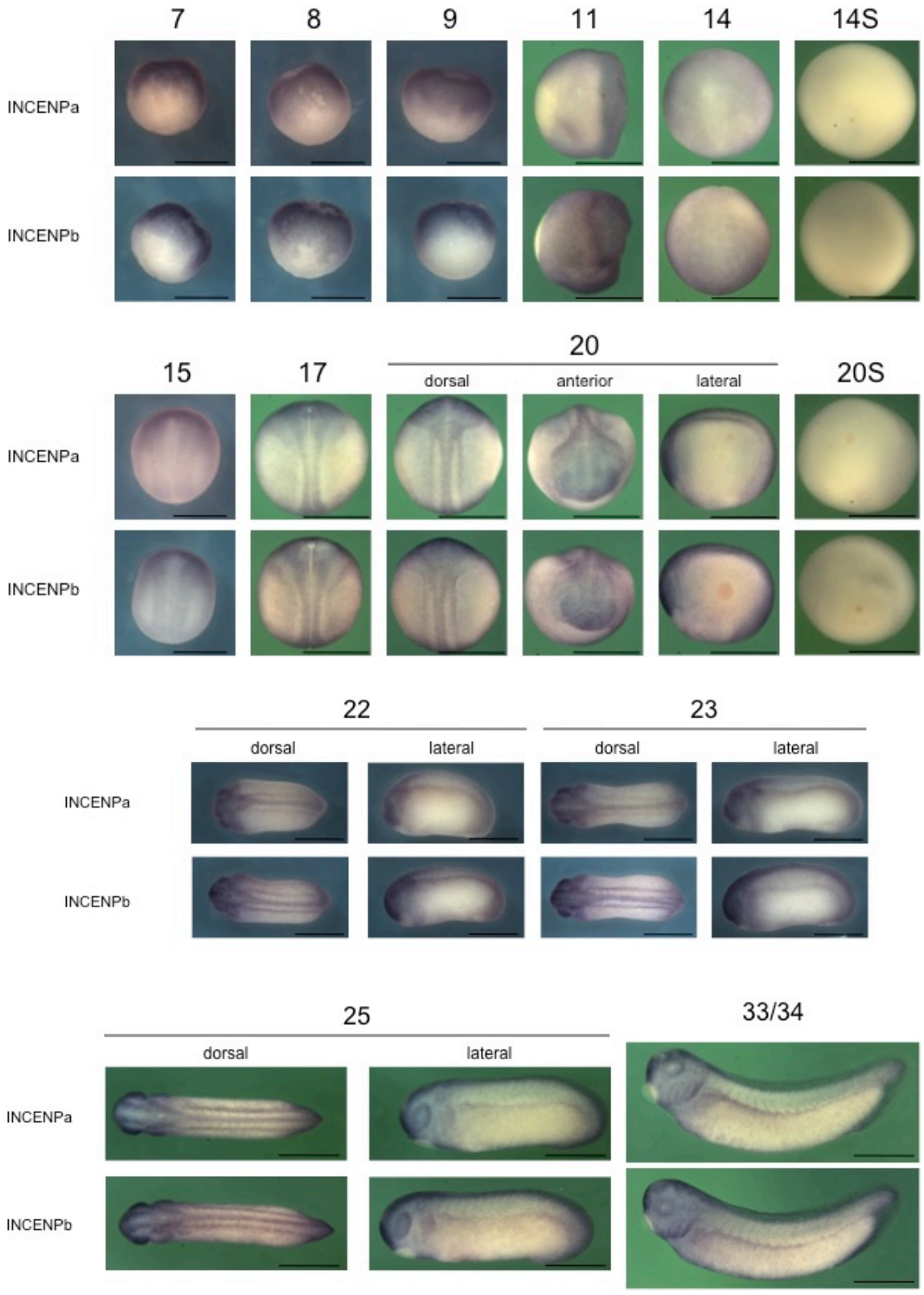
Normal NT closure

Late/mild NTD

Severe NTD

Dead embryo

Figure 13: INCENP mRNA is highly expressed in neural tissue throughout embrygenesis. *In situ* hybridization of INCENPa/b mRNA to Xenopus laevis embryos of different stages. Numbers above images indicate stage at which embryo was fixed. 14S and 20S are representative of control samples taken at each stage analyzed. Controls were probed with mRNA generated using primers sens to the INCENPa/b primers. Scale bar = 1mm.

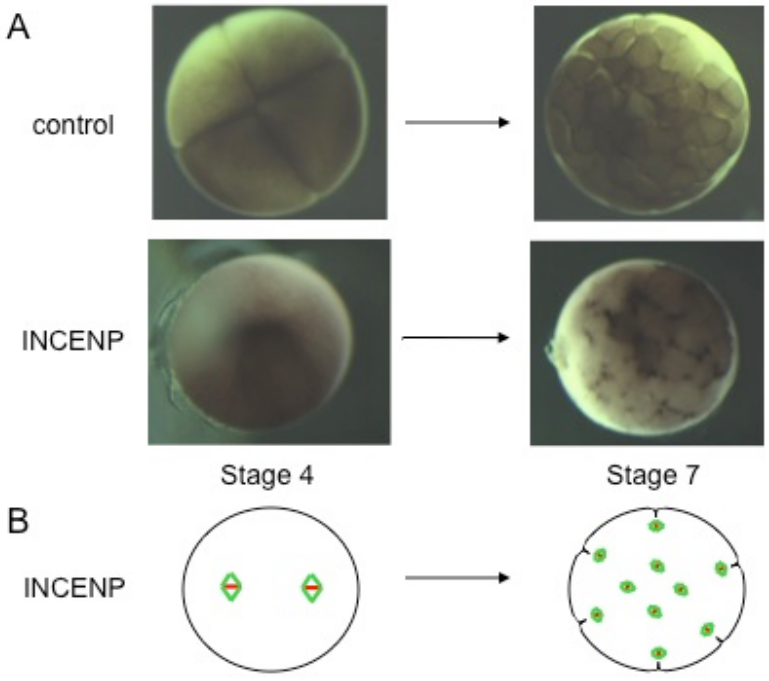

7
8
9
11
14
14S
INCENPa
INCENPb
15
17
20
dorsal
anterior
lateral
20S
INCENPa
INCENPb
22
23
dorsal
lateral
dorsal
lateral
INCENPa
INCENPb
25
33/34
dorsal
lateral
INCENPa
INCENPb

primers anti-sense to INCENPa/b coding sequences. Control probes were generated using primers sense to INCENPa/b coding sequences. Both INCENPa and INCENPb show identical expression throughout embryogenesis. INCENP is expressed in the animal hemisphere in stage 7-9 embryos. At stage 14, INCENP expression begins to increase in neural tissue, particularly in the neural plates. INCENP expression increases until stage 20, where it is highly expressed in the neural tube, head and tail regions. This high INCENP expression is maintained in these regions throughout the rest of embryo development, at least until stage 33/34.

INCENP and cytokinesis

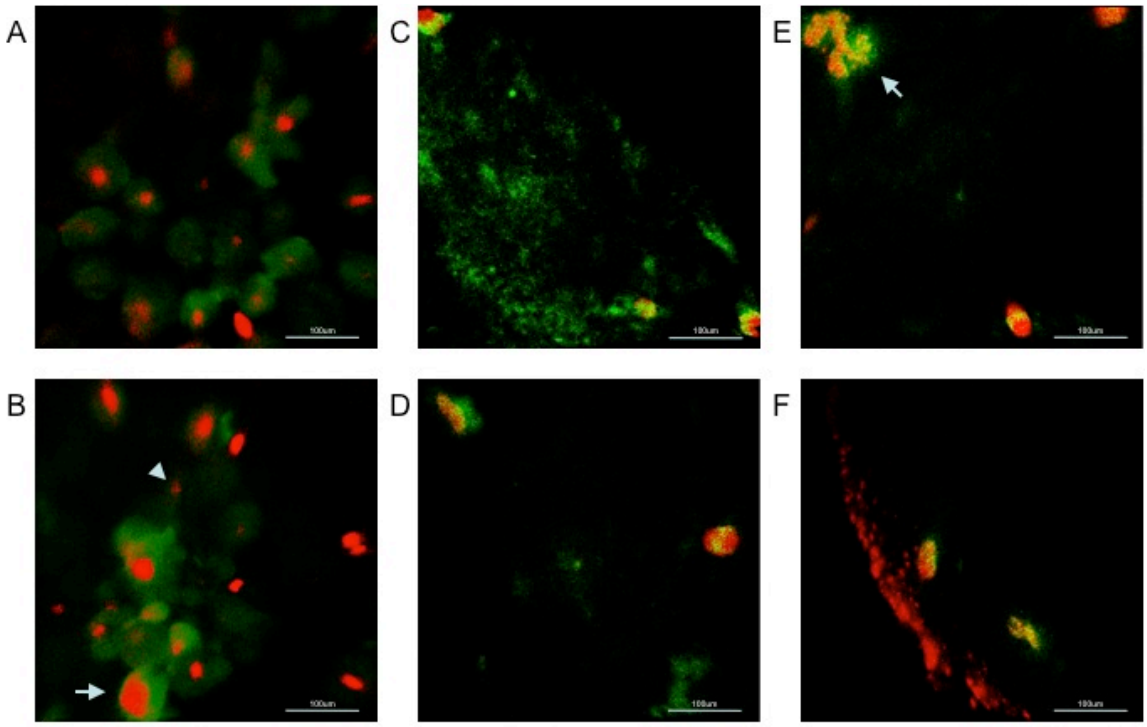
Although a certain population of $INCENP_{morpho}$ embryos (Figure 8) showed no signs of cleavage at stage 4, an interesting observation was made when they were followed beyond this stage. Figure 14A shows that at stage 7 these INCENP embryos exhibited multiple arrays of furrow-like membrane invaginations across the plasma membrane. To further investigate if this population of embryos might also be undergoing nuclear division, we fixed $INCENP_{morpho}$ embryos at stages 7, 9 and 10, and subsequently scanned these embryos for nuclei. These groups were also injected with fluorescent DNA probes at the oocyte stage, allowing visualization of nuclei. We attempted to locate nuclei by scanning the surface of embryos by confocal microscopy. The scanning depth of *Xenopus laevis* embryos using our confocal system is approximately 50um. However, we were only able to observe nuclei beginning at stage 11 by scanning the surface of the embryo. As stage 11 is after ZGA, we wanted to find nuclei at earlier time points during development. To this end, embryos were

Figure 14: INCENP$_{morpho}$ embryos form furrow array. Embryos were injected at the oocyte stage with either control (ctrl$_{morpho}$) or INCENP$_{morpho}$, matured, subjected to the host transfer technique and then fertilized. **A,** While ctrl$_{morpho}$ embryos are at stage 7, INCENP embryos only begin to form an abnormal array of furrows at the same time point. **B,** Hypothetical model where INCENP embryos continue to divide nuclei in absence of cytokinesis, resulting in a single cell containing multiple spindles. Once these spindles reach the surface of the embryo, they begin to direct the formation of furrows. Chromosomes are shown in red; microtubule spindles in green.


A
control
INCENP
Stage 4
Stage 7
B
INCENP

bisected from the animal to the vegetal pole and the interior of the embryos was scanned using a confocal microscope. Although the furrow-like invaginations were first observed at stage 7, nuclei were still not visible at this time point. However, at stages 9 and 10, multiple nuclei were observed inside the bisected embryos (Figure 15, C-F). Nuclei (identified by mChe-H2B, which labels DNA) were visible throughout the embryo but appeared to be positioned closer to the plasma membrane (visible in red in Figure 15F). Nuclei in $INCENP_{morpho}$ embryos were of comparable size and shape to nuclei in $ctrl_{morpho}$ embryos, although certain abnormal nuclei in $INCENP_{morpho}$ embryos were larger and malformed compared to $ctrl_{morpho}$ nuclei.

Figure 15: INCENP$_{morpho}$ embryos continue to divide nuclei. Scans of bisected multi-celled, ctrl$_{morpho}$ embryos (A-B) and single-celled INCENP$_{morpho}$ embryos (C-F). **A-B,** Ctrl$_{morpho}$ embryos were fixed and disected at stage 9. **B,** Arrow indicates a cell closer to the surface scanned. Arrowhead indicates a cell further from the surface scanned. **C-F,** INCENP$_{morpho}$ embryos were fixed at stage 9 (C-D) and stage 10 (E-F). **E,** Arrow indicates an abnormally large and miss formed nucleus. Multiple nuclei can be seen in both ctrl$_{morpho}$ and INCENP$_{morpho}$ embryos. red = mChe-H2B (DNA probe). green = GFP-Utrofin (actin probe). Scale bar = 100um.


A
C
E
B
D
F

Discussion

INCENP during oocyte maturation

INCENP protein levels increase during oocyte maturation (Yamamoto et al., 2008; Figure 5). Injection of $INCENP_{morpho}$ into immature (GV) oocytes inhibits this increase in INCENP (Yamamoto et al., 2008; Figure 5). Yamamoto et al. (2008) showed that this inhibition did not affect levels of cyclin B, suggesting that nuclear maturation is unaffected. In this study, they analyzed the absolute levels of cyclin B1 and B2 after GVBD. They found that in both control injected, and $INCENP_{morpho}$ injected oocytes, cyclin B1 and B2 levels declined after GVBD, and then accumulated again near metaphase II arrest. This trend follows the same decline and rise in MPF activity seen after GVBD (Ma et al., 2006; Liu et al., 2005). The authors conclude that inhibition of INCENP translation does not affect oocyte maturation. We wanted to confirm by another, more physiologically relevant, method that nuclear maturation was indeed not affected.

In order to accomplish this, we examined 1^{st} PB emission in $INCENP_{morpho}$ injected oocytes. We saw that 1^{st} PB is properly emitted in $INCENP_{morpho}$ injected oocytes (Figure 6). This confirms that injection of $INCENP_{morpho}$ does not affect the nuclear component of oocyte maturation. This would suggest that the increase in INCENP protein during oocyte maturation is dispensable for nuclear maturation.

INCENP during embryogenesis

Our hypothesis states that as a maternal factor, INCENP would be accumulated during oocyte maturation, but only necessary afterwards during development. We confirmed that the INCENP accumulated during oocyte maturation is not necessary for nuclear maturation, which consists, in part, of emission of the 1st PB. Therefore our next goal was to determine what events this accumulation of INCENP was necessary for. The main events that occur after the metaphase II arrest of the egg are fertilization, 2nd PB emission, and subsequent embryogenesis.

As mentioned above, fertilization of the egg by a sperm triggers a calcium wave that releases the egg from its metaphase II arrest. This results in completion of meiosis and emission of the 2nd PB. By pricking $INCENP_{morpho}$ injected oocytes with a glass needle, we can mimic fertilization and induce 2nd PB emission.

Figure 7 (panels 1 through 3) shows that the 2nd PB can be properly emitted in $INCENP_{morpho}$. This would suggest that accumulated INCENP is unnecessary for the processes involved in 2nd PB emission. This is true at least for the processes that occur *after* fertilization. In the experiment performed, we bypassed natural fertilization by pricking the eggs. It is possible that accumulated INCENP is required for the processes of fertilization *in vivo*, such as sperm-egg binding. This was indirectly investigated later, and we observed that embryos injected at the oocyte stage with $INCENP_{morpho}$ were still able to divide their nuclei (Figure 15). If accumulated INCENP was necessary for fertilization, then $INCENP_{morpho}$ embryos, consequently, would remain unfertilized, and nuclei would not divide. In addition,

it was discovered that INCENP$_{morpho}$ embryos showed other signs of activation, such as lifting of the vitelline membrane and contraction of the pigment on the surface of the embryo (Figure 8; "no cleavage" group). This further supports the notion that accumulated INCENP is not involved in fertilization.

Since the INCENP accumulated during oocyte maturation is not required for 2nd PB or fertilization, we hypothesized that it was necessary for embryo development. To study developmental competence, ctrl$_{morpho}$ and INCENP$_{morpho}$ injected oocytes were subjected to the host transfer technique (Heasman et al. 1991). The host transfer technique allows the eggs to acquire the jelly coat, which is necessary for fertilization. While most (82%) of the ctrl$_{morpho}$ embryos were developing normally at stage 4, only a few (4%) of INCENP$_{morpho}$ embryos were normal (Figure 8). Interestingly, 64% of INCENP$_{morpho}$ embryos showed no signs of cleaving, whereas this specific phenotype was not observed in control embryos. These data suggest that INCENP translation during oocyte maturation is essential for early embryogenesis. More specifically, the accumulated INCENP might be necessary for the initial rounds of cytokinesis in the embryo.

Rescue constructs were created and an experiment was performed to confirm that the lack of INCENP protein resulted in the defects in cytokinesis. Although rescue data are from only one experiment, certain conclusions can still be discussed. The number of embryos recovered from each group was also low in this experiment, although they remained consistent with our previous results (Figure 8). Most of the ctrl$_{morpho}$ embryos were healthy, while the INCENP$_{morpho}$ were either cleaving abnormally or not cleaving at all. Injection of HA-INCENPa or HA-INCENPb mRNA rescued the levels of INCENP protein to normal,

accumulated levels of INCENP (Figure 9). Co-injection of HA-INCENPa with HA-$INCENP_{morpho}$ at least partially rescued the effects of $INCENP_{morpho}$ on embryos. Five out of the seven HA-INCENPa embryos appeared to be cleaving normally at stage 4. The other two were cleaving abnormally. None of the INCENPa embryos displayed the prominent $INCENP_{morpho}$ phenotype of no cleavage, further supporting that HA-INCENPa can potentially fully rescue the $INCENP_{morpho}$ phenotype. Since the only embryo co-injected with HA-INCENPb mRNA and $INCENP_{morpho}$ was cleaving abnormally, it is difficult to conclude if HA-INCENPb can rescue the $INCENP_{morpho}$ phenotype. However, because INCENPa and INCENPb isoforms are so highly homologous (90%), we hypothesize that HA-INCENPb mRNA would be able to rescue the $INCENP_{morpho}$ with equal efficiency to HA-INCENPa mRNA. Although preliminary, these data do suggest that INCENP protein needs to be accumulated during oocyte maturation in order to allow for proper cytokinesis in the early *Xenopus laevis* embryo. Additional experiments would be needed to confirm this conclusion.

Efficiency of the host transfer technique

The low number of embryos recovered in the previous experiment can be explained by the nature of the host transfer technique. There is loss of the number of potential embryos during each step of the experiment. Numbers are lost during (1) stimulation with progesterone, (2) transfer into the host frog, and (3) fertilization of the eggs. First, not all oocytes are stimulated by addition of progesterone. Every frog produces oocytes that react differently to stimulation with progesterone. Generally speaking, 80 to 100% of oocytes from an individual frog will reach MII arrest after stimulation with progesterone. Secondly,

not all transferred oocytes are recovered from the frog. The MII arrested oocytes are transferred into the abdominal cavity of a frog that has already begun depositing her own eggs. The time between the transfer and deposition of the first experimental eggs takes on average 3-4h. After that, the frog will keep depositing eggs for approximately 3 more hours before she becomes exhausted from the surgery and oviposition. The frog generally stops depositing eggs before all experimental eggs are recovered. Recovery at this step is between 50-80% of transferred oocytes. Finally, not all recovered eggs (whether they are host eggs or experimental eggs) are fertilized. Eggs have a fertilization rate of between 70-90%, with the host eggs usually having a higher rate of fertilization than the experimental eggs. The final recovery of embryos, that is, the number of eggs that are fertilized compared to the number of oocytes stimulated with progesterone can vary substantially between experiments, anywhere from 20% to 50%. On average, the final recovery is approximately 40%.

The recovery of embryos is also limited by the number of oocytes that can be transferred into a host frog. A frog can hold up to 800 transferred oocytes, depending on the size of the animal. Generally, the number of oocytes stimulated with progesterone is between 100-200 per experimental group. Because the rescue experiment consisted of four groups, these groups consisted of only 50-80 oocytes/group.

INCENP and cytokinesis

A certain population of $INCENP_{morpho}$ embryos (Figure 8, "no cleavage" group) exhibited possible furrows later in development (Figure 14). This prompted the idea that these "uncleaved" embryos may actually undergo regular nuclear division (every 30 minutes) and,

at stage 7, the multiple spindles would migrate close enough to the embryo surface to induce multiple, albeit futile, cleavages (see Figure 14B for model). To test this hypothesis, $ctrl_{morpho}$ and $INCENP_{morpho}$ embryos were fixed and bisected to observe their nuclei. Although the furrow-like invaginations on the surface of $INCENP_{morpho}$ embryos were first observed around stage 7, we were unable to see any nuclei at this time point. It is possible there were too few nuclei that were not positioned close enough to the plane of bisection. However, at stage 9 and 10, multiple nuclei were observed inside the bisected $INCENP_{morpho}$ embryos (Figure 15C-F), suggesting nuclear division was still taking place.

At this point, it is unclear whether or not these nuclear divisions occur properly. For example, some nuclei were abnormally large (when compared to $ctrl_{morpho}$ nuclei) and malformed (Figure 15E, arrow). In addition, $INCENP_{morpho}$ appear to have far less total nuclei than $ctrl_{morpho}$. This would suggest that nuclei division is either slowed or arrested at some point in $INCENP_{morpho}$ embryos. It is also important to note the rather crude method of bisection. After the embryos were fixed, they were manually bisected with a sharp scalpel. The cut was made from the animal pole to the vegetal pole. Because $ctrl_{morpho}$ and $INCENP_{morpho}$ embryos are physically different, the bisection resulted in differences in the physical appearance of the cut surface. In $ctrl_{morpho}$ embryos, the individual blastomeres of the embryo are still intact. When bisected, these embryos simply “broke” apart, and blastomeres in each half of the embryo remained intact. This creates a rather “hilly”, or uneven surface to be scanned. This particularity explains why some nuclei in $ctrl_{morpho}$ appeared smaller or larger than others. After bisection, the bisected surfaces (interior of the embryo) of each embryo half were placed down on a dish to be scanned by confocal microscopy. Blastomeres that were closer to the surface (Figure 15B, arrow) scanned would

contain nuclei that appear larger than the nuclei of blastomeres located further away (Figure 15B, arrowhead). In the case of INCENP$_{morpho}$, the embryo remains a single cell. Thus, bisection of the embryo created a smoother surface. Some of the differences in size between nuclei of INCENP$_{morpho}$ could be attributed to their distance from the surface scanned, but these nuclei displayed much greater differences in size than ctrl$_{morpho}$ nuclei (compare INCENP$_{morpho}$ nuclei in figure 15E). It is more likely the larger nuclei are a result of fusion between divided nuclei, resulting from the lack of cytokinesis. In fact, our lab has previously shown that when cytokinesis is inhibited during 1st polar body emission, segregated chromosomes recongress to form a larger spindle (Ma et al., 2006).

At the very least, these data suggest that the mitotic cycle in INCENP$_{morpho}$ is not entirely disrupted, possibly not at all, and INCENP$_{morpho}$ embryos still divide their nuclei in a potentially normal manner.

This particular event in INCENP$_{morpho}$ embryos might be explained by INCENP's possible role in cytokinesis and the distance between the microtubule spindle and the plasma membrane in these embryos (see figure 12 for model). The central spindle is generally accepted to initiate the formation of the contractile ring and the furrow (Glotzer 2005). At anaphase, INCENP migrates from the spindle to the cortical site of the presumptive furrow (Earnshaw & Cooke, 1991). In INCENP$_{morpho}$ embryos, it is possible there is enough INCENP to allow proper division of chromosomes, but not enough to transmit the signal (at anaphase) from the central spindle to the cortex to initiate furrow formation. Once the multiple spindles (resulting from the lack of cytokinesis) migrate to the surface, the spindles

would then be close enough to direct the formation of furrows. It is important to note that these furrows are incomplete and the embryos eventually die.

Other evidence to support the above theory lies in the fact that $INCENP_{morpho}$ injected oocytes still emit both polar bodies (Figures 6 and 7). Polar body emission is an extreme form of asymmetrical cell division. The injection of $INCENP_{morpho}$ in oocytes inhibits translation during oocyte maturation, but does not eliminate the already present INCENP protein (stockpiled level of INCENP; Figure 5, lane 4). INCENP is necessary for chromosome segregation as well as cytokinesis (Earnshaw & Cooke, 1991). The stockpiled level of INCENP could be enough to support proper segregation of chromosomes and cytokinesis in $INCENP_{morpho}$ injected oocytes during meiosis, since the spindle is associated closely with the membrane (Ma et al., 2006; Zhang et al, 2008). However, in 1-cell embryos the spindle lies far beneath the surface of the cell. The stockpiled level of INCENP might allow for segregation of chromosomes, but this level may be inadequate to signal the formation of the cytokinetic furrow. Once the multiple spindles reach the surface, however, the low level of INCENP may finally be able to direct furrowing.

INCENP translation during oocyte maturation and embryogenesis

We have shown that INCENP translated during oocyte maturation is only necessary later in development, during embryogenesis. We have also shown that INCENP is not translated during embryogenesis, at least until stage 9 (Figure 11), which is the timing of ZGA in *Xenopus laevis* (Newport and Kirschner, 1982). Taken together, these data agree with the theory that INCENP is translated during oocyte maturation in preparation for early

embryogenesis, during which time the embryo does not have the means to translate INCENP. After ZGA, the embryo would then begin to transcribe zygotic INCENP mRNA, and translate sufficient INCENP protein to support further development of the embryo. In accordance with the latter hypothesis, injection of $INCENP_{morpho}$ into embryos should result in inhibition of INCENP translation starting at ZGA, followed at some point by embryo death. However, a peculiar result was obtained when embryos were injected at the 1-cell stage with $INCENP_{morpho}$.

An adverse phenotype in $INCENP_{morpho}$-injected embryos was only observed during neurulation, (stage 19: Figure 12), much later than expected. The specific effect on neural tube closure was also surprising. Since INCENP is involved in ubiquitous cell cycle processes (i.e. chromosome segregation and cytokinesis), we expected to see increased cell, and embryo, death in $INCENP_{morpho}$-injected embryos. However, the viability of these embryos did not seem affected (Figure 12, percentage of "dead embryos"). Rather, these embryos had an increase in neural tube defects (Figure 12, "NTD"). This data suggested that after ZGA, INCENP is only necessary for development of neural tissue. To further explore this theory, we looked at mRNA expression in *Xenopus laevis* embryos. Both INCENPa and INCENPb mRNA were highly expressed in neural tissue throughout embryogenesis, until at least stage 33/34 (tadpole; Figure 13). Since INCENPa and INCENPb are highly homologous (90%), it is likely the INCENPa probe would cross-hybridize with INCENPb mRNA, and vice-versa. Accordingly, the staining shown in Figure 13 is likely the sum of both isoforms. Taking into account the *in situ* hybridization data, it appears that after ZGA, INCENP might only be translated in developing neural tissue. The reason behind this tissue-specific expression, and neural tissue defects, is as yet unknown. It is possible that INCENP

has some specific, unknown role in developing neural tissue. However, a more conservative analysis would lead us to believe that the comparatively heightened expression of INCENP mRNA in neural tissue could be an indirect result of an increased rate of mitosis in neural tissue. Thus, the defect in NTC in $INCENP_{morpho}$-injected embryos could be due to the increased demand on mitotic proteins in neural tissue.

Regulation of INCENP translation

As mentioned above, translation during oocyte maturation and embryogenesis is tightly regulated, primarily by regulating polyadenylation of mRNA (MacNicol and MacNicol, 2010). Two sequences found in the 3' UTR of mRNA are among several sequences known to regulate, in part, this process. The Musashi binding element (MBE) is a well-studied sequence known to bind the Musashi protein, and direct early mRNA translation during oocyte maturation (Imai et al., 2001; Charlesworth et al., 2002). More recently, a novel translational control sequence (TSC) was also implicated in directing early translation of mRNA during oocyte maturation (Wang et al., 2008). Upon analysis of INCENPa/b 3'UTR, it was found that INCENPa contains a single MBE, while INCENPb contains a TCS. Curiously, neither INCENP isoform contains the cytoplasmic polyadenylation element (CPE), which is the binding site for CPEB. The CPE is an important regulatory element during oocyte maturation and is found, in conjunction with various other 3'UTR regulatory elements, in the mRNA of many major players involved in the progression through oocyte maturation, including mos, aurora A and all cyclin B variants (MacNicol and MacNicol, 2010). The CPE also has a strong influence over other 3'UTR regulatory elements and can override the early activation triggered by MBE or TCS, leading to late translation of an

mRNA. It is possible that INCENPa/b are examples of mRNA translated early in oocyte maturation. If this were to be correct, then perhaps maternal factors, specifically maternal proteins, are accumulated during oocyte maturation by regulation via early translation initiation. The single regulatory element in both isoforms of INCENP could be explained by the fact that INCENP translation is not require for progression through oocyte maturation. It is possible a more tightly controlled method of regulating translation, mediated by the interaction of multiple regulatory elements, is reserved for those proteins that are critical for nuclear maturation.

Another protein whose levels are upregulated during oocyte maturation, pericentriolar material-1 (Pcm-1), displays the same maternal factor "pattern" as INCENP. Pcm-1 is associated with the centrosomes and is implicated in assembly of centrosomes and organization of microtubules during mitosis (Balczon et al., 2002; Dammermann and Merdes, 2002; reviewed by Wang et al., 2008). Wang et al. (2008) showed that early translation of Pcm-1 during oocyte maturation is regulated by its TCS found in the 3'UTR of its mRNA. Balczon et al., (2002) demonstrated that an anti-Pcm-1 antibody injected into murine embryos leads to cell-cycle arrest, while injection into oocytes appears to have no affect on oocyte maturation. Although not directly assessed, these data strongly suggest that Pcm-1 translation during oocyte maturation is a maternal factor, and is important for embryo development, similar in principle to INCENP. Therefore, in order to stockpile enough specific protein to allow for proper embryogenesis, it is possible the oocyte initiates translation of these specific proteins early in oocyte maturation, via MBE- and TCS-mediated mechanisms.

Conclusion

We have shown that INCENP is not translated during early embryogenesis. In addition, using morpholino against INCENP mRNA, we showed that the INCENP protein accumulated during oocyte maturation is essential for early embryogenesis in *Xenopus laevis*. Since the accumulated INCENP is dispensable for nuclear maturation, we propose that the translation of INCENP protein during oocyte maturation is a maternal factor of vertebrate embryogenesis.

This demonstrates that certain proteins accumulated during oocyte maturation may misleadingly seem unimportant because they do not appear to be a component of what was traditionally thought to be a "healthy oocyte". However, the definition of what represents a "healthy oocyte", is continuously expanding to include all factors that can have a critical impact on the development of the embryo. The discovery and analysis of novel maternal factors will lead to a better understanding of the necessary biochemical ingredients that generate life. Additionally, the identification of novel maternal factors may lead to new methods of screening healthy oocytes for use in assisted reproductive technologies.

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Curriculum Vitae

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Sept 2004 – May 2008	Queen Elizabeth II Scholarship, Government of Ontario
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Presentations

Leblond, G. and Liu, X.J. *De Novo* translation of the mitotic protein INCENP during oocyte maturation is required for embryogenesis. University of Ottawa BMI graduate seminar day. Feb 17th, 2010. Ottawa, Canada.

Leblond, G., Liu, X.J. De novo translation of the mitotic protein INCENP during oocyte maturation is required for embryogenesis. Ottawa Hospital Research Institute Research Day, Nov 27th, 2009. Ottawa, Canada.

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