University of South Bohemia in České Budějovice

Faculty of Science

Cloning useful recombinant cDNA/ mRNA constructs to study cell cycle and Hippo-signalling in preimplantation mouse embryos

---

Bachelor Thesis in Biological Chemistry

Fabian Moser

České Budějovice, 2019

Supervisor: Assoc. Prof./ doc. Alexander W. Bruce Ph.D.
Department of Molecular Biology and Genetics

Biblical reference

Moser, F., 2019: Cloning useful recombinant cDNA/ mRNA constructs to study cell cycle and Hippo-signalling in preimplantation mouse embryos. Bc. Thesis, 43p., Faculty of Science, University of South Bohemia in České Budějovice, Czech Republic.

Annotation

The aim of this thesis was the generation of an *in vitro* transcription (IVT) plasmid vector containing a cDNA insert for an EGFP-Tubulin-6 fusion protein, studying the effect of non-phosphorylatable YAP1(S112A) on preimplantation mouse embryo blastomere localisation and generation of Securin-GFP mRNA for microinjection in early mouse embryo blastomeres.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in a publicly accessible part of the IS STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 09.12.2019 .....

Fabian Moser

ACKNOWLEDGEMENTS

First and foremost, I want to thank my supervisor and head of the Laboratory of Early Mammalian Developmental Biology (LEMDB), doc. Alexander W. Bruce Ph.D. for giving me the opportunity to work on very exciting and interesting projects. I really appreciate your trust in my experimental work, you letting me work so freely, your professional expertise and your patience with me!

I want to thank my lab colleagues Giorgio Virnicchi Ph.D. and Pablo Bora Ph.D. Thank you for dedicating your time, answering my questions when I was stuck in my work and most importantly for ensuring such a positive and bright atmosphere in the laboratory!

Further, I want to show my gratitude to prof. RNDr. Libor Grubhoffer, CSc., for his very caring and dedicated guidance through this bachelor's program Biological Chemistry.

I would like to thank my study colleagues, with whom I spent three years of my life and made friends for life with!

Finally, I thank my family. I could not have finished my studies without you!

Abstract

During regular somatic cell division (mitosis), the previously replicated genetic material is equally divided between two daughter cells. As replicated chromosomes condense, and two sister chromatids are drawn towards two opposing cell poles by the microtubules (MT) of the spindle apparatus. To study MT formation, localisation and mitotic spindle dynamics, a modified Tubulin variant cDNA incorporating an enhanced green fluorescent protein (EGFP) tag, EGFP-Tubulin-6, was inserted into an appropriate cloning vector that would allow *in vitro* translation of mRNA for study on mouse preimplantation stage embryos. After initial failed attempts to insert the EGFP-Tubulin-6 cDNA into the preparatory vector pRN3P, using restriction enzyme mediated ligation, the gene was successfully TA-cloned into the commercial vector pGEM-T-Easy. A GFP tagged mRNA for the spindle assembly checkpoint (SAC) protein Securin was also produced. In preimplantation mouse embryo development, cells of the developing embryo (blastomeres) give rise to not only the foetal progenitor, but also precursors of extra-embryonic supportive tissues. During mouse preimplantation embryo development, three distinct lineages are established in two 'cell fate decisions'. In the first cell fate decision the placement of cells within the embryo is crucial, as an outer position sustains individual blastomere polarity, while inner cells remain apolar and activate the 'Hippo-signalling pathway' promoting pluripotency by preventing (via phosphorylation dependent mechanism) the transcriptional co-activator YAP to enter the nucleus. Accordingly, in polar outer cells YAP becomes nuclear and ensures TE cell fate. Here, a mutated non-phosphorylatable YAP variant (S112A) mRNA was microinjected into single 2-cell stage embryo blastomeres and the localisation and cell number of the microinjected cell clone (versus the non-microinjected clone of the same embryos) was determined by confocal microscopy. Surprisingly, the microinjected blastomeres did not show a significant difference in commitment to the TE or ICM lineages, at the developmental stages studied.

Concise Project Summary

Early mammal development begins with the fusion of the sperm with the egg, resulting in a totipotent zygote. This zygote (1-cell stage embryo) then undergoes a series of cleavage cell division, but the overall volume of the embryo does not increase. At the 8-cell stage a morphological process known as compaction begins, whereby individual blastomeres (an archaic name for cells of the early embryo) establish new junctions between each other and maximize their contact area. As development proceeds, newly created blastomeres can reside encapsulated inside or on the outside of the developing embryo, via sensing their relative areas of cell-cell contact. Starting at the late 8-cell stage and continuing into subsequent stages, outer cells gain an intra-cellar polarity and will ultimately contribute to the trophectoderm (TE – the embryo derived precursors of the placenta). As such, polarised outer cells, supress the Hippo-signalling pathway (ensuring the nuclear accumulation of the transcriptional co-activator, YAP) that in turn promotes the establishment of the transcription factor driven gene expression programme that actively promotes TE differentiation (and inhibits pluripotency). Conversely, apolar inner cells are sustained in a pluripotent state that ensures the inner cell mass (ICM) is capable of contributing to all foetal/-embryonic tissues post-implantation (although they can also contribute via the second differentiation and extraembryonic tissue known as the primitive endoderm/ PrE; a precursor cell population that yields the yolk sac. Accordingly, in inner cells activated Hippo-signalling ensures YAP1 is phosphorylated and retained in the cytoplasm, thus promoting pluripotency and supressing TE differentiation. Such delineation of the outer, polarized and differentiating TE from the inner, apolar and pluripotent ICM is often referred to as the "first cell fate decision". Once the developing embryo has reached the 32-cell stage, a fluid-filled cavity forms within (displacing the ICM to one pole) and the resulting embryonic structure is known as the blastocyst and is primed for hatching /uterine implantation; this process is concomitant with a "second cell fate decision" by which ICM cells either differentiate into PrE or retain pluripotency as the so-called epiblast (EPI) population (precursor of the foetus).

To successfully undergo all the above described morphological and cell fate specification/differentiation events, the blastomeres must faithfully divide and partition their replicated DNA/ chromosomes, resulting in two genetically identical daughter cells in the mitotic phase of the cell cycle (*i.e.* mitosis). The mitosis itself is characterized by five phases (Prophase, Prometaphase, Metaphase, Anaphase and Telophase), but it is during the middle "metaphase" stage, that the condensed and replicated chromosome pairs (*i.e.* chromatids) are

aligned on the medial plane of the cell by interaction with spindle associated microtubule (MT) fibres, that attach to the central kinetochore structures of the chromatids. The spindle MT fibres are made from tubulin, that is assembled from α and β variants. Prior to the onset of the "anaphase" stage of mitosis, whereby sister chromatids are segregated to the opposing poles of the spindle via their kinetochore attached MT fibres, a spindle assembly checkpoint (SAC) system controls if all of those MT-kinetochore connections have been appropriately established, actively retarding anaphase onset if not. A central component of SAC system involves the protein securin, which indirectly prevents separation of the sister chromatids, via inactivating the Separase enzyme complex required to maintain cohesion, until its proteolytic degradation at the onset of metaphase exit/ anaphase. Thus, Securin must be degraded before the cell can proceed to the next phase (*i.e.* anaphase) of mitosis.

The aim of this thesis was divided into three parts. The main goal was the sub-cloning of a cDNA insert encoding a EGFP-Tubulin6 fusion protein into a new vector from which mRNA could be derived via *in vitro* transcription (IVT) and microinjection into the embryo blastomeres/ oocytes. After an initial failure to ligate the required insert in the desired pRN3P vector, the insert was successfully inserted into the alternative pGEM-T-Easy vector, using the method of TA-cloning. Consequently, IVT derived mRNA was derived from the newly synthesised plasmid construct. A secondary aim was to utilise IVT to produce mRNA from a pre-existing construct encoding HA-YAP1(S112A), which yields a variant of YAP protein (tagged with a N-terminal HA-epitope tag) that is non phosphorylatable and can thus potentially not be retained in embryonic blastomere cytoplasm, irrespective of the status of Hippo-pathway activity of the cell and is predicted to drive cells to commit to TE lineage. Thirdly, as a side project recombinant and IVT-derived mRNA for a Securin-GFP fusion protein was produced for injection into mouse embryo blastomeres, to permit study of SAC dynamics; though time constrictions ultimately did not allow for successful microinjection.

Glossary of abbreviations

APC/C	Anaphase-promoting-complex/cyclosome
BSA	Bovine serum albumin
Bub	budding uninhibited by benzimidazole gene (mouse)
Cdc20	Cell-division cycle protein 20 gene (mouse)
Cdk1	Cyclin-dependent kinase 1 gene (mouse)
Cdx2	Caudal type homeobox 2 gene (mouse)
CENP-E	Centromere protein E
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotid triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPI	Epiblast
F-actin	filamentous actin
Gata3	GATA binding protein 3 gene (mouse)
GE	Gel electrophoresis
hCG	human chorionic gonadotropin
HPLC	High performance liquid chromatography
ICM	Inner cell mass
IVT	*In-vitro* transcription
KSOM	Potassium-simpex media for mouse embryo culture
Lats	Large tumor suppressor kinase
MCC	Mitotic checkpoint complex
MOPS	3-(*N*-morpholino)propanesulfonic acid
Mud	Mitotic-arrest deficient gene (mouse)
NFW	Nuclease free water
NFW	Nuclease-free water
PAR1	Partitioning defective mutant (mammalian homolog) protein 1
PCR	Polymerase chain reaction
Ph/Ch/IAA	Phenol:Chloroform:Isoamyl Alcohol
PMSG	Pregnant mare’s serum gonadotropin
PrE	Primitive endoderm
RNA	Ribonucleic acid

SCRIB	Scribble planar cell polarity protein
Sec	Securin gene (mouse)
TAE	Tris acetate EDTA
TE	Trophectoderm
TGF-α	Transforming growth factor alpha
TJP	Tight junction protein
UTR	Untranslated region
Yap1	yes-associated protein gene (mouse)
γ-TURC	γ-tubulin ring complex

TABLE OF CONTENT

1 Introduction

1.1 The cell cycle

The life of cells can be divided in four different stages; M, G_1, S and G_2 – *described below* (Figure 1). Fundamentally however, these can be classified in major two parts that are defined as mitosis (during which replicated chromosomes are separated and cell division is completed – equivalent to the "M" stage) and the remaining interphase (G_1, S & G_2). The interphase is the time between two cell divisions and begins with the unpacking of the condensed chromosome structures (post-cell division), increased metabolism and the cell growths; as no replication of the DNA occurs, this G_1-phase is called a gap phase. The beginning of DNA replication marks the start of the following S-phase, where the genetic information is replicated and the chromosomes gain a second chromatid. When the replication process is finished, the cell enters a second gap phase, to grow and prepare for separation in two daughter cells (during M-phase), the G_2-phase (Bayrhuber et al. 2010).

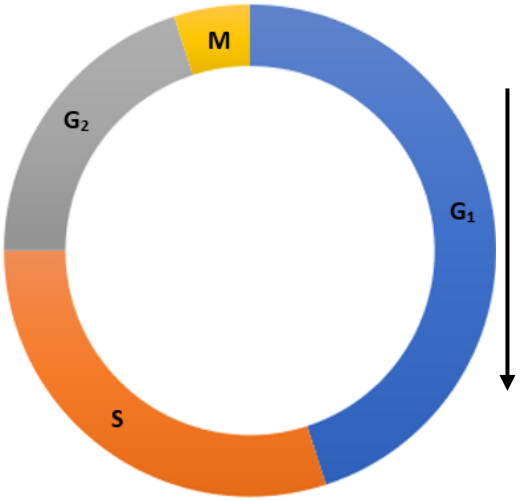

Figure 1 | **Schematic of the phases of the cell cycle.** G_1 = Gap phase 1, S = Synthesis, G_2 = Gap phase 2, M = Mitosis

Mitosis is the step in the cell cycle where the cell divides into two genetically identical cells and because this is a very complex procedure, mitosis itself is further divided into five sub phases (Figure 2). It begins with prophase, where chromosomes gather cohesion and thus change from a diffuse to a condensed state of appearance, becoming visible by light microscopy, and the mitotic spindle apparatus (needed to separate replicated sister chromatids into the two resulting daughter cells) also begins to form. The following prometaphase marks an important step in spindle assembly, as the nuclear envelope is shattered into vesicles, which are to be ultimately divided between the daughter cells. This allows the centrosomes that form the bipolar spindles and initiate microtubule fibre polymerisation, to occupy the central region of cytosol. Consequently, microtubule fibres emanating from opposing spindle pole centrosomes are then able to attach to the kinetochores within the centromeric region of sister chromatids. During the subsequent metaphase stage the condensed chromosomes congress up on the equatorial plane between the spindles (known as the metaphase plate). At this point a

complex checkpoint system, known as the spindle assembly checkpoint (SAC) ensures all sister chromatid kinetochores are attached to microtubule fibres emanating from opposing spindle poles. Thus, the SAC directly inhibits the so-called anaphase-promoting-complex (APC/C) from initiating the separation of sister chromatids (*i.e.* the next stage of mitosis, known as anaphase) until all chromatid kinetochores are appropriately attached to the spindle pole microtubules; such APC/C inhibition thus prevents the activation of the enzyme securin, which eventually leads to the separation of the sister chromatids by breaking their cohesion with each other (See chapter 1.1.2). Next, in anaphase, the sister chromatids are differentially drawn towards the opposing spindle poles, by shortening of the microtubules that are docked to their respective kinetochores.

In the final telophase stage, when the chromatids have reached the poles, the nuclear membrane reforms, the chromatids decondense and the original cell is cleaved into two daughter cells in a process known as cytokinesis. The final result are two daughter cells, each comprising exactly the same genetic information (O'Connor C. 2008).

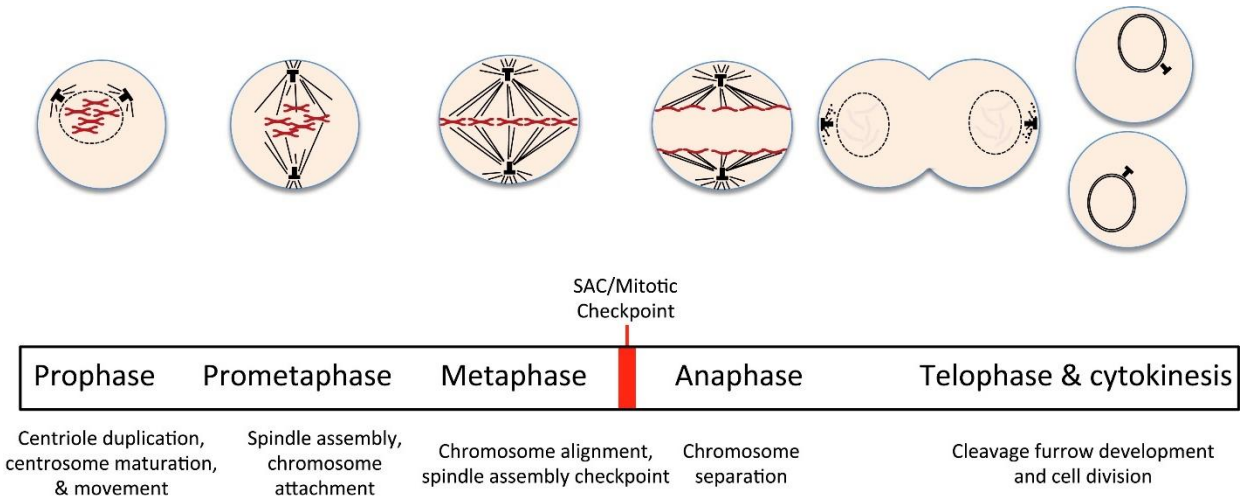

Figure 2 | **Summary of mitotic phases.** Mitosis starts with Prophase, where the previously diffuse chromosomes condense and is followed by the Prometaphase where the chromosomal envelope is shattered, and spindle assembly starts. In the metaphase, the chromosomes align on the metaphase plate between the two spindle poles and microtubules (MT) attach to the chromatids central regions (kinetochores). Correct MT attachment is governed by a Spindle assembly checkpoint system (SAC), which regulates onset of the subsequent Anaphase, where the sister chromatids are drawn towards the opposing poles. In the final telophase stage, the nuclear membrane reforms and finally the cell cleaves into two daughter cells, in a process called cytokinesis (taken from Dominguez-Brauer et al. 2015).

1.1.1 Microtubule attachment in mitotic metaphase

During metaphase, the chromosomes are aligned in the medial plane, between the two poles of the spindle apparatus; *i.e.* on the metaphase plate. Microtubule fibres emanate from opposing poles of the spindle apparatus and attach to specialized protein complexes residing at the central region of the mitotic chromatid. These kinetochores consist of a trilaminar stack of plates and a mesh of fibres, known as the fibrous corona. The fibrous corona and the outer plate harbour the majority of the microtubule interacting proteins, like *CENP-E* or dynein, as well as SAC checkpoint proteins, important for sensing/reporting the proper attachment of the microtubules and thus ultimately promoting the onset of the subsequent anaphase. The inner plate is composed of chromatin with specialized histone structure, as it is adjacent to the centromere. Composition of the middle plate is unknown (Chan et al. 2005).

The spindle itself consists mainly of microtubules, hollow cylindrical structures of multiple parallel protofilaments. These comprise of α-/β-tubulin heterodimers arranged in a head-to-tail configuration. α-tubulin is exposed at the so-called minus/- terminal ends of the protofilament fibres with β-tubulin being present at the plus/+ end (Nogales et al. 1999). The slower growing minus end extends into the centrosome with the faster growing plus ends being oriented towards the chromosomes/chromatid pairs aligned on the metaphase plate. The resulting lattice of the microtubules also contain kinesin-like proteins and dynein, ATP driven motor proteins, allowing movement and sliding of the spindle microtubules fibres relative to each other (regulating spindle length) and the recognition of critical cargoes for transportation, required to maintain spindle structure, integrity and polarity (Desai and Mitchison 1997).

The centres for microtubule initiation, growth and organisation are the centrosomes. Each consists of two centrioles enclosed by pericentriolar material, generating microtubule arrays, that elongate the plus ends outward. The pericentriolar material contains mainly γ-tubulin. This specialized tubulin forms the ring complexes γ-TuRC, which catalyse the formation of new microtubules. The high demand of microtubules during mitosis is covered by recruitment of multiple γ-TuRCs to allow rapid production of microtubules. The two opposing poles of the bi-polar mitotic spindle apparatus each contain one centrosome that were derived after centrosome duplication in the preceding S-phase (Walczak and Heald 2008).

1.1.2 Spindle-Assembly-Checkpoint (SAC) system

The incorrect separation of sister chromatids can lead to cancerous growth, dysfunctional cells or cell death; or in developmental contexts, aborted development or severe morphological malformations. Thus, as every cell needs a precisely defined number of chromosomes, the exacting distribution of the sister chromatids is the crucial purpose of mitosis. Therefore, the correct attachment of the spindle microtubules emanating from opposing spindle poles to the appropriate sister chromatid kinetochores is strictly monitored (it is critical that the kinetochores of sister chromatids are attached to microtubules derived from opposing spindle poles) and the onset of the next mitotic stage (anaphase) is inhibited incorrect or absent attachments are detected (Straight 1997; Amon 1999).

As illustrated in Figure 3, identification of the appropriate attachment of sister chromatid kinetochores is controlled by the group of mitotic-arrest deficient genes (*Mad*), *Mad1*, *Mad2* and *Mad3* (=*Bubr1*) and the budding uninhibited by benzimidazole genes (*Bub*), *Bub1* and *Bub3* (Musacchio and Hardwick 2002). The checkpoint system is centred around the regulated release of active separase, an enzymatic protein that binds to the so-called cohesins (that hold sister chromatids together and prevent their separation, even when under spindle derived tension) and hydrolyses their ring structure. (Hauf et al. 2001). *Mad* and *Bub* proteins work in diverse ways to target *Cdc20*, a cofactor subunit of the anaphase-promoting complex/cyclosome (APC/C) and thus regulate its activity in a manner dedicated by the completion of appropriate microtubule-kinetochore attachments. APC/C itself performs a poly-ubiquintination function to target its substrate proteins, for proteasomal destruction. One of the target proteins is securin (SEC*)*, which normally interacts with and inactivates free separase, rendering it unable to cleave the cohesin ring structure. (Peters 2006). Hence, sister chromatid cohesion is maintained until the SAC proteins (*Mad* and *Bub* proteins) permit activation of the APC/C, via *Cdc20*, and the destruction of SEC resulting in activated Separase. Another APC/C target protein is cyclin B; when APC/C targets Cyclin B for proteosomal destruction it is no longer available to bind and activate its cognate partner *Cdk1* (the master mitotic cyclin dependent kinase), thus promoting exit from mitosis. Thus, any mitotic cell is unable to continue into anaphase unless both securin and *Cdk1* consent. In summary, *Mad* and *Bub* build a mitotic checkpoint complex (*MCC)*, that consist of *BUB3*, *MAD3* and *CDC20*. This complex inhibits APC/C, hindering it from ubiquintination of securin/SEC and cyclin B (*i.e.* the SAC). When spindle assembly is correctly established, the cascade of the checkpoint system allows activation of Separase and the promotion of anaphase (Musacchio and Salmon 2007).

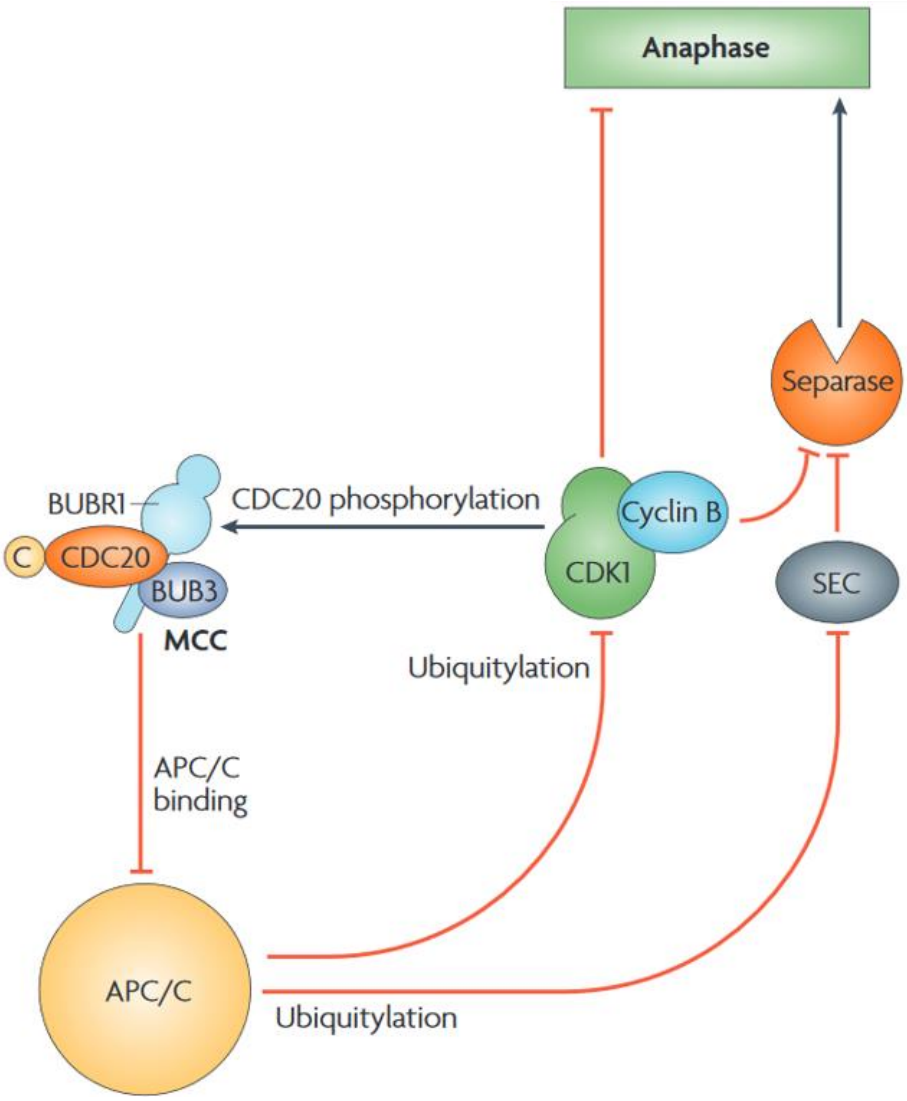

Figure 3 | **Partial feedback pathway of the spindle assembly checkpoint system.** During metaphase, multiple proteins converge to form the mitotic checkpoint complex (*MMC*). *MMC* formation is stimulated through cyclin dependent kinase 1 (*CDK1*) and through other pathways not shown in this figure. The *MMC* consists of the budding uninhibited by benzimidazole protein *BUB3*, the mitotic-arrest deficient protein *BUBR1* (=*MAD3*) and the cofactor cell-division cycle protein *CDC20*. This complex inhibits the anaphase promoting complex/cyclosome (*APC/C*), which marks securin (SEC*)* and cyclin B for degradation by polyubiquitination. This leads to the release of separase, degradation of the cohesin ring bonding around the sister chromatids and to execution of the anaphase (taken from Musacchio and Salmon 2007).

1.2 Early mouse development

Mouse development starts with ovulation of the egg and its subsequent fertilization by a sperm in the female oviduct/ reproductive tract. Accordingly, the resulting fertilized egg, the so called zygote, indicates the starting point of the preimplantation developmental period (Mihajlović and Bruce 2017). Fertilization is thus followed by serial cleavage cell divisions,

characterized by the absence of interphase growth (Figure 4). During this time the total cytoplasmic volume of all blastomeres (cells) of the developing embryo remains constant to that initially provided in the zygote; thus, whilst the conceptus proceeds through successive cleavage cycles, the individual cells are getting progressively smaller (Aiken et al. 2004). Note that during the second meiotic division of the oocyte, a small polar body arises (containing a haploid complement of maternal chromosomes) but eventually degenerates post fertilisation, as preimplantation development proceeds and is thought not to play any significant role in embryogenesis (Villena and Sapienza 2001).

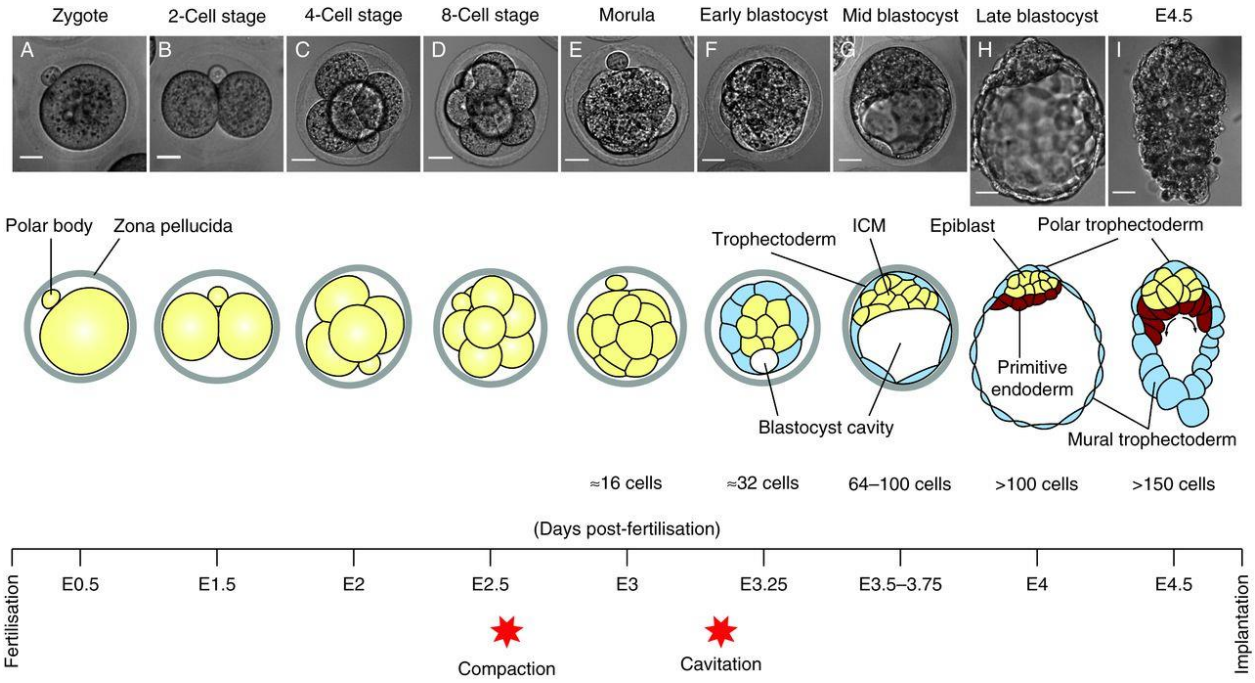

Figure 4 | **Preimplantation mouse embryo development.** The development of the mouse embryo starting from the moment of fertilization of the female egg to the time of implantation. Also indicating the time period in days (Saiz and Plusa 2013).

1.2.1 1st cell fate decision

Until the 8-cell stage of embryo development, each blastocyst remains totipotent, and can thus form any tissue, including extra-embryonic tissues (*i.e.* TE and PrE derivatives); although it is only zygotes and individual 2-cell stage embryo blastomeres that on their own can sustain full embryonic development to birth (Kelly 1977; Chazaud and Yamanaka 2016). Between the 8- and 32-cell stage, the embryo undergoes a series of specialised cell divisions, that can yield either outer (with contactless apical domain surfaces) or inner (totally encapsulated within the

embryo; with maximal cell-cell contacts) daughter cells. Thus, a given 8-cell stage (or outer 16-cell stage) blastomere can divide asymmetrically to produce an outer cell (complete with an apical domain and basolateral surfaces in contact with its neighbouring cells) and an inner cell. Alternatively, such cells can divide symmetrically, where both daughter cells remain in the outer compartment of the embryo. Inside cells may only arise from asymmetric division from outer cells, while outer cells can develop only by symmetric divisions (Fleming T. P. 1987). Cells residing on the outside of the 32-cell stage embryo (also called the late morula or early blastocyst stage) are progenitors for the extraembryonic trophectoderm (TE), eventually developing exclusively into the embryonic component of the placenta. Inner cells will contribute to the inner cell mass (ICM), initially remaining pluripotent until they either differentiate to provide the second extraembryonic tissue called the primitive endoderm (PrE) or achieve a state of primed naïve pluripotency as epiblast (EPI) cells (the progenitor cells for all the tissues of the later embryo/foetus). Thus, after the 32-cell stage the cells of the TE and ICM are committed to their respective fates and can no longer divide asymmetrically. This process is referred to as the first cell fate decision. (Johnson 1981; Fleming T. P. 1987; Yagi et al. 2007; Saiz and Plusa 2013).

In the committed TE/ICM lineages, different transcription factors determine the potency of the individual cells. TE cells lose their pluripotency progressively, by expression of the transcription factor gene *Cdx2*, which is in turn is regulated by the transcription factor TEAD4. *Cdx2* is responsible for the suppressed expression of pluripotent transcription factors genes, that are only normally expressed within inner cell, including *Oct4* and *Nanog*. Indeed, within the ICM, the expression of the pluripotent *Oct4* and *Sox2* transcription factor genes promotes the expression of *Nanog*, which in turn preserves pluripotency in ICM cells (Strumpf et al. 2005; Jedrusik et al. 2008).

1.2.2 Compaction and Polarisation

When the embryo reaches the 8-cell stage of preimplantation development, the cells undergo morphological changes. While before individual blastomeres were only in direct contact via small areas of their surface, owing to their spherical shape, a process called compaction ensures they flatten and maximize their contacts with neighbouring cells. Compaction is governed by the establishment of new intercellular junctions and is crucial for the subsequent correct morphogenic formation of the blastocyst (Ducibella T. and Anderson E. 1975; Hyafil 1980). Accordingly, the formation of adherens junctions, gap junctions and tight junctions are established along the basolateral membranes of 8-cell stage blastomeres. The protein components for such junctions are already present in the cells (thus, their transcription and translation is not required) and comprise namely, E-Cadherin (CDH1) and the catenins CATNA and CATNB plus the thight-junction proteins (TJP1 and TJP2) (Sheth et al. 2008). The junctions are progressively established until the 32-cell stage, forming the so-called late morula structure (prior to cavity formation and expansion that is the hallmark of the forming blastocyst stage; for which they provide the necessary structural integrity). As shown in Figure 5, compaction also allows for intra-cellular polarisation (expanded upon below) of 8-cell stage blastomeres, that is then maintained in the outer cell populations in the morula/blastocyst (Wiley and Eglitis 1981; Sefton et al. 1992; Hyenne et al. 2005).

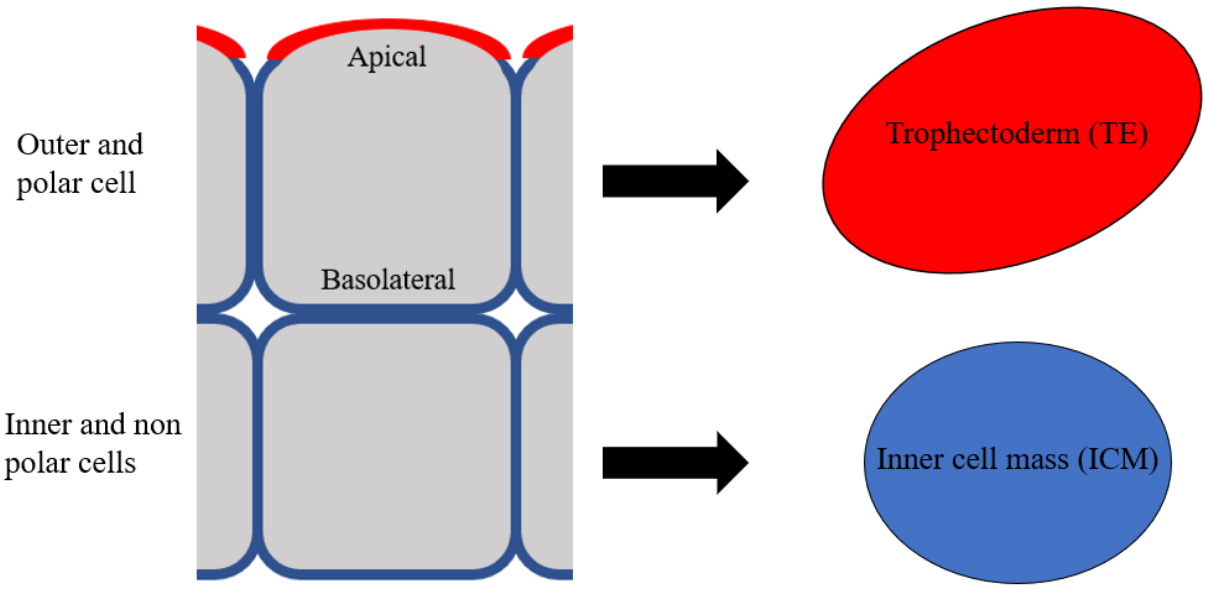

Figure 5 | **Polarization of apical and basolateral cells by compaction.** After compaction a polarity arises at outer cells, as those experience less cell-to-cell contact and thus have an apical and a basolateral domain. Outer blastomeres will eventually go on developing the trophectoderm (TE) and inner cells, which only have basolateral domains, remain neutral, retain their pluripotency and contribute ultimately to the inner cell mass (ICM)

At the late 8-cell stage, a process of intra-cellular polarization yields two different membrane domains. The contact less surface is defined as the apical domain, whereas the regions of the membrane in contact with neighbouring blastomeres are designated the basolateral domains; therefore from the 16-cell stage outer cells remain polar by virtue of inheriting both apical and basolateral domains, but inner cells are apolar and comprise only the original basolateral domains (Johnson M. H. and McConnell M. L. 2004). Apical domains are characterized by the presence of a specific protein complex containing the polarity factors *PAR3*, *PAR6* and apical protein kinase C. Furthermore, they are enriched in a high number of microvilli, F-actin and Rho GTPases. Basolateral domains (in both polar outer cells and the cell membranes of inner cells) are also enriched in a different class of polarity factors (including PAR1 and SCRIB) (Yamanaka et al. 2006; Chazaud and Yamanaka 2016; Mihajlović and Bruce 2016).

1.2.3 Hippo signalling pathway

The Hippo signalling pathway is characterized by a complex set of signalling cascades that can ultimately regulate cell growth, differentiation, proliferation and apoptosis. Not only does it play a key role in embryonic development, but it is also a critical pathway studied in the context of cancer research (Pan D. 2007, 2010; Kim and Jho 2014). During early embryogenesis, the Hippo signalling pathway plays a fundamental role in the commitment of blastomeres to either TE or ICM cell fates; these are summarised in Figure 6.

Indeed, it is now known that maximal cell-cell adhesion/contact typical of inner cells activates Hippo signalling, which in turn supresses the accumulation of the transcriptional co-activator protein Yes-associated-protein (YAP), in the cell nucleus. Thereby the transcription factor TEAD4 is indirectly supressed from activating transcription of TE-related genes (*e.g. Cdx2*). Moreover, the absence of active TEAD4 transcriptional complexes also permits expression of the ICM specific transcription factors *Oct3/4*, *Nanog* and *Sox2*. The outer residing blastomeres have fewer cell to cell contacts and thus experience weaker Hippo signalling (that is also directly inhibited by the presence of an apical domain and the enriched apical polarity factors), allowing YAP to accumulate in the nucleus and enable TEAD4 to activate its target genes. As TEAD4 is activated, it induces expression of *Cdx2* and *Gata3*, that finally promote differentiation of the blastomere to the TE (Hirate et al. 2013).

1.2.4 Yes-associated protein 1 (YAP1)

The *Yap1* gene encodes the transcription coactivator YAP. It complexes with TEAD4 to form a transcriptional complex that potentiates the expression of TE specific genes, such as *Cdx2* and *Gata3*. It acts only in the cell nucleus, but can also be found translocated/sequestered in the cytoplasm (Yagi et al. 1999; Hirate et al. 2013). As such, YAP localisation is phosphorylation dependent and it is naturally found in the cell nucleus, although when phosphorylated, it is retained in the cytoplasm and thus becomes inactive. Studies have shown, that the expression of recombinantly engineered non-phosphorylatable YAP, as employed in this thesis, can potentially increase CDX2 protein expression, resulting in a loss of blastomere pluripotency and commitment to the TE lineage (Dong et al. 2007; Yagi et al. 2007; Hao et al. 2008; Hirate et al. 2013).

It has also been shown, that localization of YAP is regulated by the serine/threonine-protein kinases of the *Lats* gene family. Mammals exhibit two variants of *Lats* kinase: *Lats1* and *Lats2*. Expression of high levels of LATS1/2 are known to cause the phosphorylation of YAP1 and drive its localisation to the cytoplasm (Nishioka et al. 2009).

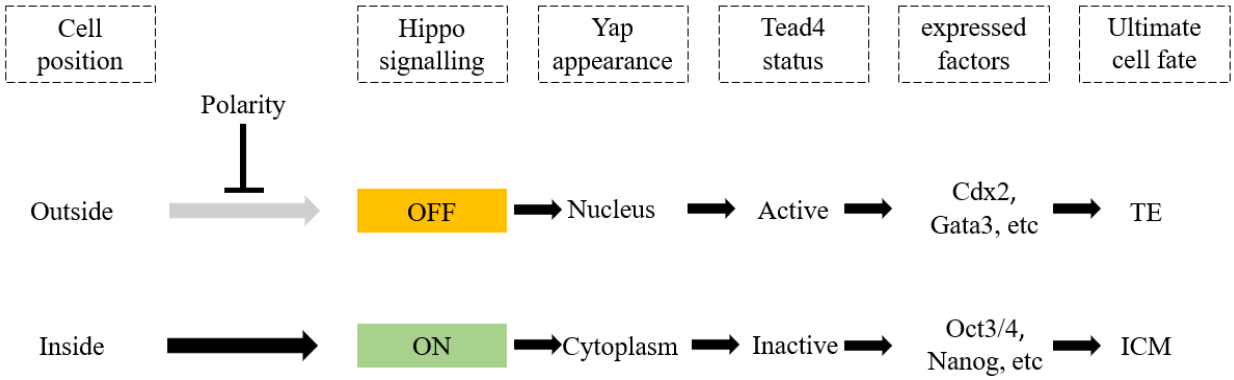

Figure 6 | **Hippo signalling effects in mouse preimplantation stage embryos.** The effect of hippo signalling is determined by the cell position and thus the polarity of the cell, hippo signalling influences the localization of *YAP*, following the activation or inactivation of *TEAD4*. Availability of functional *TEAD4* then determines the transcription factors expressed, which then determine the fate of the cell.

1.2.5 Blastocyst development

When the preimplantation embryo reaches the blastocyst stage (comprising of 32-cells) the embryo begins to grow in overall size. This process is mediated by the establishment of a fluid filled cavity on the inside of the embryo structure/morphology. In the preceding late morula stage, at least one variant of Na/K-ATPase becomes active, allowing a

trans-trophectoderm movement of Na^+-ions (Watson and Kidder 1988). This ion gradient between the extra-embryonic space and the cavity allows the transfer of water by osmosis that provides the means for the cavity to grow. The tight-junctions formed in the outer TE cells, that were established during compaction, provide a seal to prevent leakage and sustain cavity expansion. Furthermore, growth factors like *TGF-α* and *EGF*, also facilitate expansion of the cavity, by stimulating Na/K-ATPase activity (Watson 1992).

1.2.6 2nd cell fate decision

During the first cell fate decision, cells of the preimplantation embryo diverge to form either the TE and lose their pluripotency or populate the ICM and maintain their pluripotency. According to their relative position with reference to the forming blastocyst cavity, the blastomeres of the ICM are segregated into two further cell lineages, resulting in the formation of the differentiating primitive endoderm (PrE) or the primed and pluripotent epiblast (EPI). As such, cells adjacent to the cavity by the late blastocyst stage (when the cavity is maximally expanded – Figure 4) comprise the PE and most inner and encapsulated cells of the ICM comprise the EPI.

Three models have been put forward, on how ICM cells choose their fate. The first and earliest established model is quite similar to the first cell fate decision and states that the cells naturally adjacent to the cavity are triggered and will go on to form the PrE, while the more inner cells of the ICM yield the EPI (Dziadek 1979). Another, more recent, model suggest cells of the ICM spontaneously/ stochastically give rise to PrE and EPI progenitors that then actively sort into the correct position by the late blastocyst (Chazaud et al. 2006). The third model proposes a social mobility paradigm, whereby the second cell fate decision has its foundations at the 8- to 16-cell stage transition, where a first wave of asymmetric divisions generates the majority of the EPI cells and the second wave of asymmetric divisions (from 16- to 32-cell stages) provides the majority of the PrE. According to this latter cell history related model, those blastomeres, which are not in the right position within the ICM can execute one of three options: i) change their gene expression to fit their position, ii) move to the appropriate position by active cell sorting, or iii) undergo apoptosis (Zernicka-Goetz et al. 2009).

2 AIMS of the study

This thesis project consisted of two main aims and one side project. The main aim consisted of the generation of an *in vitro* transcription (IVT) plasmid vector construct containing an EGFP-Tubulin-6 cDNA as an insert, to allow derivation of mRNA to be microinjected in to mouse embryo blastomeres. The main target vector was to be pRN3P, as it harbours useful untranslated regions (UTRs), that prevent degeneration of the transcribed mRNA after microinjection into preimplantation mouse embryo blastomeres. With this fluorescently labelled protein construct, the localisation of Tubulin in the cell, as well as spindle assembly and dynamics, could be studied (yet outside of the confined aims of this bachelor's project).

Secondly, a non-phosphorylatable variant of the transcriptional coactivator *YAP1* gene was to be transcribed from a the pre-existing plasmid vector construct pcDNA3.1-pA83:HA-YAP(S112A). The resulting mRNA was to be microinjected into single 2-cell stage embryo blastomeres and the effect on the localization (and hence fate: TE versus ICM) of the respective daughter cells determined by confocal microscopy.

Finally, as a side project, mRNA of pre-existing pRN3P-Securin-GFP was to be transcribed. The consecutive microinjection into preimplantation mouse embryo blastomeres, to study the localisation and appearance of the spindle assembly checkpoint (SAC) protein Securin, were a prospect for future projects.

3 Materials and Methods

3.1 Cloning vectors

To conduct the following experimental procedures, the pre-existing preparatory cloning vector pRN3P and the commercially available vector pGEM-T-Easy (Promega) were used. Figure 7 and 8 indicate the vector maps of the respective empty vectors.

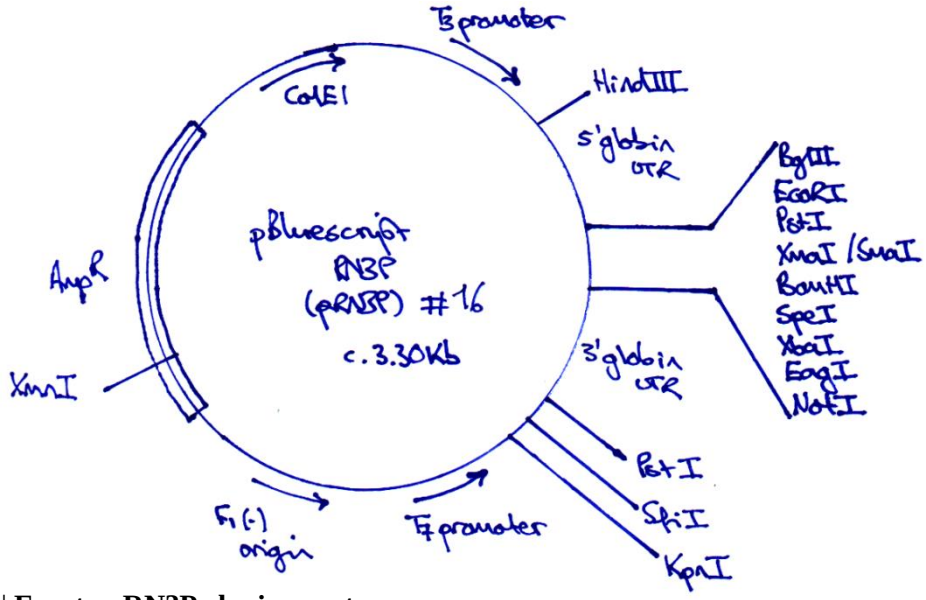

Figure 7 | **Empty pRN3P cloning vector map.**

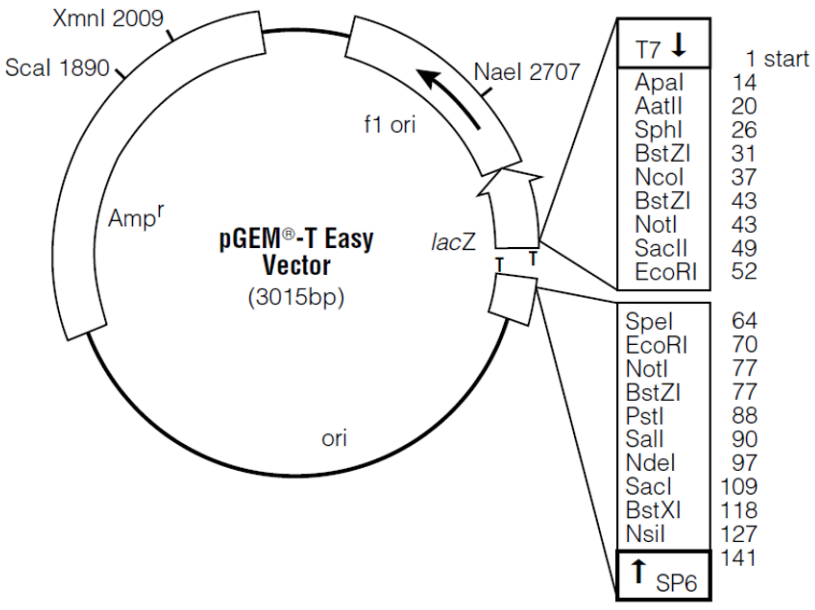

Figure 8 | **Empty linearized pGEM-T-Easy cloning vector map.** (Promega 2015)

3.2 Plasmid cloning

3.2.1 LB Media

To make LB bacterial culture media, 10 g bactotryptone, 5 g yeast extract and 10 g NaCl was added to 800 mL distilled H_2O and the pH is adjusted to 7.5 using concentrated sodium hydroxide (NaOH) and the volume made up to 1 L. In the case of making LB agar plates, 15 g of agar were dissolved, prior to the step that increased the volume to 1 L, and then the volume was increased to 1 L and the mixture was sterilized by autoclaving.

3.2.2 Gel electrophoresis (GE)

A mixture of 1 g of agarose (AgaroseServa) and 100 mL 1x TAE buffer was dissolved in a microwave. To the molten mixture, 2 µL ethidium bromide [which inserts itself into the DNA double strand, and allows visualization of the DNA, through exposure to U.V. light (Wahl et al. 1970)], was added. This was poured to a gel slab mould, together with a comb to introduce wells for the introduction of the samples post cooling. Next, 1x TAE buffer solution was poured around the gel, to fully cover it in the gel electrophoresis tank/apparatus.

Desired quantities of DNA/RNA samples were mixed with 6x purple loading dye (NEB) to achieve 1x concentration and loaded into single wells, as was a reference ladder containing fragments of DNA/RNA of known size (*i.e.* 1 Kbp+ ladder, in the first well) A voltage of 80 V was applied across the gel to allow differential migration of the loaded DNA/RNA species, according to their molecular weight, through the agarose towards the positive electrode. The resulting gels were then observed under UV light and reference photographs taken.

3.2.3 Plasmid transformation

A number of obtained plasmid DNAs (harbouring desirable inserts or to act as recipient vectors) were required for this project. These were transformed into *E. coli* in order to amplify and purify them in sufficient quantity for downstream molecular biology/ recombinant DNA experimentation. For this, 3 µL of ligation mixture of pRN3P/pGEM-T-Easy vector with EGFP-Tubulin-6 insert, was placed into a 2 mL vial with 15 µL competent *E.coli* cells and allowed to rest for 30 minutes on ice. Next, the suspension was heat shocked in a water bath at 42 °C for precisely 55 seconds and placed back on ice for 10 minutes. To the culture 500 µL SOC-Media (SERVA) is added and the suspension then warmed in a water bath at 37 °C for 60 minutes. The mixture was centrifuged at 400 rpm for 5 minutes at room temperature and the supernatant discarded. The pellet was then resuspended in 100 µL H_2O (HPLC-grade, VWR)

and applied to an agar (SERVA) plate, which was pre-prepared with an antibiotic in it, here mostly ampicillin (100 ng μL^{-1}) was used. The cells were allowed to grow in an incubator at 37 °C, after about 16 hours the plate was visually controlled for growing bacterial colonies. Growing colonies were then picked up from the plate using a pipette tip and streaked out on another agar plate in a sterile manner, to allow the bacteria further growth in an incubator (37 °C, 16 hours) and subsequent storage at 4 °C for further experiments.

3.2.4 Plasmid extraction

For the extraction of plasmids from transformed *E-coli* cells, QIAGEN Plasmid Kits were used. For smaller scale plasmid extraction, the Mini version was used, for larger scale extractions the Midi variant was employed. First, bacterial cultures were harvested from glycerol stocks or from previously transformed *E. coli* from plasmid transformation and were grown in 2 mL (Mini) or 25 mL (Midi) LB Media in an incubator at 37 °C for about 16 hours. The mixture was centrifuged at 6000 x g for 15 minutes at 4 °C and resuspended in 0.3 mL (Mini) or 4 mL (Midi) of resuspension Buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA), where 100 µg mL^{-1} RNase A was added beforehand. Then, an identical volume of lysis Buffer P2 (200 mM NaOH; 1 % SDS (w/v)) was added and the suspension mixed by gently inverting six times at room temperature. Next, an identical volume of precooled neutralisation Buffer P3 (3.0 M Potassium acetate, pH 5.5) was added and the mixture was again inverted six times to mix before 5 minute incubation on ice (Mini); 15 min (Midi). The solution was centrifuged at 16000 g for 10 minutes at 4 °C. Next, the included QAIGEN-column tip was equilibrated with 20 mL (Mini) or 100 mL (Midi) Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15 % Isopropanol (v/v); 0.15 % Triton X-100 (v/v)) by direct application to the filter surface and letting it empty through gravity flow. The sample solution was added onto the filter and permitted to flow through under gravity. The filter was then twice rinsed with 2 mL (Mini) or 10 mL (Midi) wash Buffer QC (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15 % Isopropanol (v/v)). Elution of the DNA was achieved, by application of 0.8 mL (Mini) or 5 mL (Midi) of preheated (65 °C) elution Buffer QF (1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 10 % Isopropanol (v/v)). The purified plasmid DNA was then precipitated using 0.56 mL (Mini) or 3.5 mL (Midi) of isopropanol and centrifugation at 16000 x g for 30 minutes at 4 °C. The supernatant was discarded and the pellet washed with 1 mL (Mini) or 2 mL (Midi) 70 % Ethanol (Penta) solution. Finally, the resulting plasmid DNA was dissolved in 30 µL H_2O (HPLC-grade, VWR) and the concentration determined using a NanoDrop U.V. spectrophotometer.

3.3 Polymerase chain reaction (PCR)

PCR was used to generate insert cDNA (*i.e.* EGFP-Tubulin-6) from the pre-existing plasmid pEGFP-Tubulin-6 and to conduct colony PCR (Section 3.5) to screen for successful ligation products, using the primers stated in Table 4. These primers also included the required restriction sites for the restriction enzymes *BamHI and NotI*, which are crucial for the applied restriction enzyme mediated ligation method to clone the insert into the vector pRN3P. To conduct polymerase chain reactions, the Phusion high-fidelity PCR master mix Buffer (25 mM TAPS-HCl, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ß-mercaptoethanol, 200 µM dNTPs, 15 nM primed M13 DNA; New England Biolabs) system was used for reactions requiring high precision (*e.g.* generation of cDNA inserts for plasmid cloning) and for more general applications (*e.g.* colony PCR) the AmpiGene Taq Mix (AMPIGENE Taq Polmerase, 6 mM $MgCl_2$, 2 mM dNTPs, enhancer, stabilizers; Enzo) was used. Table 1 indicates the reaction volumes used. Finally, the mixture was processed in a PCR cycler with the temperature cycle indicated in Table 2 for 30-40 cycles between denaturation and elongation phase.

Table 1 | Reaction composition and volume for PCR

Component	Producer	Volume / µL
Phusion-HF PCR MM *or* AmpiGene Taq Mix	New England Biolabs Enzo	25
Tuba1B_NEGFP_a	Sigma	2.5
Tuba1B_NEGFP_s	Sigma	2.5
DMSO	Sigma	1.5
Plasmid solution		ca. 12 ng
H_2O (HPLC-Grade)	VWR	fill to 50

Table 2 | Temperature program for the PCR

Phase	Timespan / minutes	Temperature / °C
Initialization	2	96
Denaturation	0.7	96
Annealing	1	58
Elongation	2.5	72
Final elongation	10	72

3.4 Colony PCR

To confirm the successful ligation of inserts (EGFP-Tubulin-6) into the desired plasmid cloning vector pRN3P/pGEM-T-Easy (post-transformation), colony PCR was performed. Colonies contained on bacterial transformed agar plates of interest were picked using with a pipette tip and placed in 50 µL H_2O (HPLC-Grade; VWR). Then the cells were lysed/destroyed by heating to 96 °C on a PCR block for 15 minutes (to liberate the plasmid DNA). The mixture was put on ice for 5 minutes, followed by PCR (Section 3.4) as described in Table 3 and the primers indicated in Table 4, which were specific to the insert sequence (EGFP-Tubulin-6), with the temperature program seen in Table 2. Agarose gel electrophoresis was then performed, and the size/ molecular weight of the generated PCR products compared to the DNA ladder (1 Kbp+). The result is compared to a positive and a negative control PCR reaction, that was also run on the same gel.

Table 3 | Reaction composition and volumes for colony PCR

Component	Producer	Volume / µL
AmpiGen Taq Mix	Enzo	2.5
Primers (s:a [1:1])		0.5
Template colony		2

Table 4 | Primers used for pNGFP-Tubulin6

Primer	Sequence	Producer
Sense	GACTATggattcATGGTGAGCAAGGGCGAGGAGCTG	Sigma Aldrich
Antisense	GACTATgcggccgcTTAGTATTCCTCTCCTTCTTCCTC	

3.5 Organic extraction

Removing impurities from any prepared nucleic acid preparations is important; particulary when proceeding in a succession of molecular biological techniques and ultimately deriving a product that will be introduced into living cells. In this study organic extractions followed by ethanol precipitations were used. The steps are similar when working with DNA or RNA, but the Phenol-Chloroform-Isoamyl Alcohol-Mixture (25:24:1; Sigma) was set at different pH; Ph/Ch/IAA at pH = 6.7 was used for RNA, while pH = 8 for DNA. The Ph/Ch/IAA mixture was always saturated with 10 mM Tris containing 1 mM EDTA. For RNA nuclease free water (NFW) was used for pellet resuspension, for DNA HPLC-grade H_2O.

To perform the extraction, the sample was brought to a volume of300 µL by addition of H_2O (HPLC-grade; VWR or NFW; NEB). The Ph/Ch/IAA mixture was added to double the volume. Then the mixture was vortexed and centrifuged for 2 minutes at 16000 x g. The upper aqueous phase containing the DNA/RNA sample was transferred to a fresh vial, where an equal volume of chloroform was added, vortexed and similarly centrifuged. To the removed aqueous phase 30 µL $NaAc_2$ (sodium acetate) and 750 µL 100 % Ethanol (Penta) were added. The sample was allowed to precipitate overnight at 4 °C and the solution was centrifuged at 16000 x g for 15 minutes, and the supernatant removed from the pelleted DNA/RNA and discarded. 500 µL of analytical grade 70 % ethanol was then added and similarly centrifuged to extract excess salt from the DNA/RNA pellet. The supernatant was then removed, the pellet was allowed to air dry and was then resuspended in the desired amount of H_2O (HPLC-grade of NFW)

3.6 *In-vitro* transcription (IVT)

To convert the plasmid cloned cDNA inserts (EGFP-Tubulin-6, HA-YAP(S112A) & Securin-GFP) to recombinant mRNAs for microinjection into embryonic blastomeres, transcription reactions were performed. This was performed using the *in-vitro* transcription (IVT) technique. First, the plasmid with the desired cDNA sequence in it was linearized, using a restriction enzyme (Table 6). The reaction volumes and composition are stated in Table 5 and the respective restriction enzyme and buffer for every experiment can be seen in Table 6, the respective buffer composition in Table 12. The reactions were carried out at 37 °C in a water bath for 4 hours.

Table 5 | Reaction composition and volumes for vector linearization for IVT

Component	Producer	Volume / µL
Vector with insert		to achieve ca. 3000 ng
Restriction enzyme		1
Buffer		5
H_2O (HPLC-grade)	VWR	to 50

Table 6 | Buffers and restriction enzymes used for the respective constructs

Construct	Buffer	Restriction Enzyme	Producer
pGEM-T-EGFP-Tubulin-6	NEBuffer 3.1	Nsi1	New England Biolabs
pcDNA3.1-HA-YAP(S112A)	CutSmart Buffer	Xba1	
pRN3P-Securin-GFP	CutSmart Buffer	Sfi1	

After digestion, 0.5 µL of the reaction mixture were run on agarose electrophoretic gels besides a control reaction without the restriction enzyme, to verify successful linearization of the construct. The reaction volume was increased to 300 µL H_2O (HPLC-grade; VWR) and an organic extraction and ethanol precipitation was performed, as described in section 3.7.. The resulting linearized plasmid DNA pellet was resuspended in 7 µL H_2O (HPLC-grade) and the concentration determined using a NanoDrop U.V. spectrophotometer. The DNA was then transcribed to mRNA using either the MEGAScript-T7 or the MEGAScript-T3 kits from Ambion (according to the provided protocols, chosen after the available promoter sequence on the individual plasmid constructs) for 4 hours at 37 °C. Table 7 and 8 indicate reaction volumes and components. Another organic extraction and ethanol precipitation was performed.

Table 7 | MEGAScript-T7 reaction

Component	Volume / µL
Linearized construct	ca. 500 ng
ATP solution	2
GTP solution	2
CTP solution	2
UTP solution	2
10x T7 Buffer	2
T7 Enzyme Mix	2
NFW	to 20

Table 8 | MEGAScript-T3 reaction

Component	Volume / µL
Linearized Construct	ca. 500 ng
NTPcap mix	10
10x T3 Buffer	2
T3 RNA polymerase	2
NFW	to 20

Following the completion of the IVT reaction, 1 µL TURBO DNase (New England Biolabs) was added, to remove the plasmid DNA template, and the reaction was carried out at 37 °C for 20 minutes. Results are confirmed by agarose gel electrophoreses. This was then followed by another organic extraction and ethanol precipitation to purify the IVT derived mRNA. Finally, the IVT mRNA was poly adenylated using the Poly (A) Tailing Kit from Ambion (according to the provided instructions). Table 9 provides the reaction components and volumes. The poly adenylation reaction was carried out at 37 °C for 1 hour.

Table 9 | Reaction composition and volumes for poly adenylation

Component	Volume / µL
Sample RNA	20
25 mM MnCl2	10
10 mM ATP	10
5x E-PAP Buffer	20
E-PAP Enzyme	4
NFW	36

3.7 DNA digestion

For separation of plasmid vectors from inserts, or to linearize empty vectors and prepare inserts for ligation, restriction enzymes were used to digest the plasmid and insert samples. Digestion was performed at 37 °C for 4 hours in a water bath. Table 10 reports the general composition of a digestion reaction, while Table 11 indicates the precise constructs and the respective restriction enzymes used. All enzymes and buffers used in this thesis are produced by NEB. Table 12 shows the buffer composition for all used digestion buffers.

Table 10 | Reaction composition and volume for plasmid digestion

Component	Volume / µL
Sample plasmid	ca. 5000 ng
Restriction enzyme	1
Restriction enzyme 2	1
10x Buffer	5
10x BSA	5
H_2O (HPLC-grade)	to 50

Table 11 | Restriction enzymes and Buffers used for the respective samples for digestion

Sample	Restriction enzymes	Buffer
pRN3P-e Tubulin-GFP	BamH1 & Not1	NEBuffer 2.1 or CutSmart Buffer
pGEM-T-EGFP-Tubulin-6	BamH1 & Not1	CutSmart Buffer
pGEM-T-EGFP-Tubulin-6	Bgl2 & Nsi1	NEBuffer 3.1
pRN3P	BamH1 & Not1	NEBuffer 3.1

Table 12 | Composition of Buffers for plasmid digestion

Buffer	NaCl / mM	Tris-HCl / mM	MgCl2 / mM	BSA / µg ml^{-1}	Potassium acetate / mM	Tris-acetate / mM	Magnesium acetate / µg ml^{-1}	pH
NEBuffer 2.1	50	10	10	100				7.9
NEBuffer 3.1	100	50	10	100				7.9
CutSmart Buffer				100	50	20	10	7.9

3.8 Gel extraction

It was sometimes necessary to remove two different size DNA fragments from a single preparation (*e.g.* after plasmid digestion to retrieve only the linearized species). This was achieved by resolving their differential mobility in agarose gels and physically excising the pieces of gel containing the desired fragments. These were then purified from the agarose using the GenElute Gel Extraction Kit (Sigma). Briefly, the gel was weighed and placed in a 2 mL micro-centrifuge tube, three times the volume of gel solubilization solution was added and the suspension incubated at 60 °C for 10 minutes. A binding column was prepared by rinsing it with 500 µL of column preparation solution. When the gel was fully dissolved, 1 gel volume of 100 % isopropanol was added to the sample and the mixture applied onto the binding column and spun through on a bench top microfuge for 1 minute. The column was the similarly washed with 700 µL wash solution and the DNA eluted with 50 µL preheated (65 °C) elution solution into a fresh vial (and quantified).

3.9 Ligation reaction

Recombining a plasmid vector and an insert is achieved via *in-vitro* ligation. Here, reactions involving the PCR derived EGFP-Tubulin-6 insert and the cloning vector pRN3P were conducted. T4 DNA ligase (Roche) was used (according to the manufacturer's instructions). The supplied ligation buffer (10x concentration) contained 660 mM Tris-HCl, 50 mM DTT, 50 mM $MgCl_2$ and 10 mM ATP at a pH of 7.5. Table 13 indicates the reaction volumes for performed ligation reactions at 4 °C for 16 h.

Table 13 | Ligation reaction volumes

Component	Volume / µL
Insert	ca. 1000 ng
Vector	ca. 300 ng
10x Ligation buffer	1
T4 DNA ligase	1
H_2O (HPLC-grade)	to 10 µL

3.10 TA-cloning

An alternative method for introducing an insert into a plasmid vector, especially for hard to ligate DNA fragments, is the TA-cloning method. In the present study, this method was applied to insert EGFP-Tubulin6 into the commercial and linearized Promega pGEM-T-Easy

vector, that contains 1-basepair 3'- 'T (deoxythymine)' overhangs. The necessary 3'-'A (deoxyadenosine)'-overhangs on the insert, required for subsequent ligation, were created by incubating the insert DNA with Taq-Polymerase (Enzo), that catalyses the addition of the non-templated 'A (deoxyadenosine)' deoxynucleotide; the reaction was performed at 72 °C for 20 minutes (Table 14).

Table 14 | 3'A-overhang reaction

Component	Volume / µL
Template	16 (ca. 2200 ng)
10 mM dATP	1
2x AmpiGene Taq Mix	25
H_2O (HPLC-grade)	8

The modified insert was then purified using the QIAquick PCR Purification KIT (QIAGEN), according to manufacturer's instructions. Briefly, 250 µL of buffer PB was added to the A-tailing mixture and applied to a QIAquick column, that was placed inside a 2 mL microfuge tube, and centrifuged at 16000 x g for 1 minute. The column was then washed with 750 µL wash buffer PE and similarly centrifuged. The column was then transferred to a fresh 1.5 mL microfuge tube and 50 µL H_2O (HPLC-grade; VWR) was added to elute the DNA, after centrifuge under the same conditions described above.

Once the A-tailed insert was purified, the concentration of the template was determined using a NanoDrop U.V. spectrophotometer and a ligation reaction (using linearized pGEM-T-Easy as vector) was prepared. The ligation reaction is carried out at 4 °C for 16 h, the details are stated in Table 15.

Table 15 | Ligation reaction for TA-cloning

Component	Volume / µL
pGEM-T-Easy Vector	ca. 50 ng
EGFP-Tubulin-6 (Insert)	ca. 50 ng
T4 DNA Ligase	1
2x Ligation buffer	5
H_2O (HPLC-grade)	to 10

3.11 Embryo collection

Mouse embryos were collected from F1 hybrid females (CBA/W x C57BL6). Briefly, super-ovulation was induced, by injection of 7.5 IU pregnant mare's serum gonadotropin (PMSG) at 16:00 hours. 7.5 IU of human chorionic gonadotropin (hCG) were injected exactly 48 hours later and mating was permitted with F1 hybrid males. After successful mating, judged by the presence of a vaginal sperm plug, female and male mice were separated. After 1.5 days post-hCG injection, the female mice were sacrificed by cervical dislocation and the oviducts removed by dissection. Embryos were liberated from the oviduct by micro-dissection and collected in warmed (37 °C) M2-medium, where they are washed in consecutive drops, by moving from one drop to the next, using a glass pipette and mouth suction pipette. Embryos were then transferred into a series of KSOM growth media drops overlaid with sterile mineral oil (culture plates containing KSOM drops were pre-equilibrated in a 37 °C incubator with 5 % CO_2 atmosphere) to wash and ultimately culture in the terminal ~20 µL KSOM drop, until the desired developmental stage (in a 37 °C incubator with 5 % CO_2 atmosphere).

3.12 mRNA microinjection

In this thesis project, EGFP-YAP1(S112A) mRNA was microinjected into one cell of 2-cell stage embryos. Accordingly, the mRNA was diluted to 400 ng μL^{-1} and micro-injected using the FemptoJet microinjection system on an Olympus IX80 inverted fluorescence microscope equipped with Leica micromanipulators. At the same time, H2ARFP mRNA (150 ng μL^{-1}) was also co-microinjected to positively identify for clonal progeny of the microinjected blastomere, after further *in-vitro* culture (*e.g.* on the confocal microscope after fixation).

3.13 Confocal microscopy

To observe the EGFP-YAP1(S112A) mRNA microinjected embryos, after *in-vitro* culture to the desired developmental stage and 20 minutes fixation in 4 % paraformaldehyde solution, an Olympus FlouView FV10i confocal microscope was employed. As the microinjected YAP1(S112A) mRNA comprises a fusion with enhanced green fluorescence protein (EGFP) it is easily distinguishable from the control H2A-RFP co-microinjected mRNA, carrying red fluorescence protein (RFP). Embryos were observed, as a series of z-stack images, at the 16 or 32-cell stage. The lasers of the confocal microscope were set to predetermined defaults to excite the fluorescent proteins and the DNA stained with DAPI (present in the mounting solution).

For image analysis, the program FV10-ASW 3.1 Viewer (Olympus) was used, where cells exhibiting expression of the mutated YAP1(S112A) were counted against the non-expressing cell clones and their position (inside or outside the embryo) was noted. A similar analysis was performed on a control set of microinjected embryos that had been microinjected with H2A-RFP encoding mRNA alone.

4 Results and Discussion

4.1 Molecular cloning of EGFP-Tubulin-6

4.1.1 Cloning attempts into the plasmid vector pRN3P

The main aim of this thesis project was to insert a cDNA fragment encoding a Tubulin-GFP fusion protein, comprising a human derived Tubulin component, that is 100 % identical to the mouse orthologue, into the plasmid vector pRN3P. Successful cloning of the insert into pRN3P would permit derivation of Tubulin-GFP mRNA using *in vitro* transcription (IVT) from a T3 RNA polymerase promoter upstream of the insertion site, that would also introduce stabilising 5' and 3' untranslated regions (UTRs) from the frog β-globin locus (ensuring robust stability and translation when microinjected into mouse embryo blastomeres). Therefore, a pre-existing plasmid clone containing a Tubulin-GFP cDNA insert (pEGFP-Tubulin-6) was obtained to provide a source of DNA from which the insert could be amplified (incorporating the necessary restriction enzyme recognition sequences, *BamH1* and *Not1*, required for eventual sub-cloning into pRN3P).

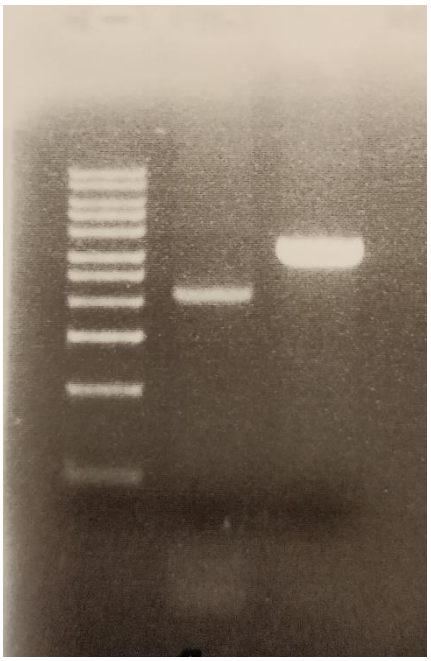

Figure 9 | **Linearization of pRN3P and digestion of Tubulin-GFP on an agarose gel.** Both products show the correct size (Tubulin-GFP: 2.3 kb; pRN3p: 3.3 kb) after initial digestion next to a 1Kbp+-ladder (Bottom to top [kb]: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10)

Figure 9 shows, that digestion of pRN3P and pEGFP-Tubulin6 yielded the expected size products (note the pRN3P *BamH1* and *Not1* digestion yielded one single stranded band without

any nicked or uncut plasmid species being present, confirming successful linearization). The digested pRN3P and PCR derived EGFP-Tubulin-6 insert were then subject to DNA ligation and the resulting ligation mixture transformed into *E. coli* and plated on LB agar plates containing ampicillin as the selectable marker. However, although the digestions seemed successful, no transformed bacteria colonies were obtained; despite multiple repetitions.

To verify that the two restriction enzymes used (*BamH1* and *Not1*) were working correctly and each were digesting the insert/plasmid vector, each enzyme was tested individually and at multiple different concentrations. This was because the linearized pRN3P plasmid vector observed in Figure 9 could have theoretically arisen if only one enzyme had been working; however, under such circumstances it would not have been possible to clone the EGFP-Tubulin-6 insert into the pRN3P vector, due to lack of sticky/cohesive end complementarity between one end of the insert and the corresponding end of the linearized target recipient vector. Conclusion of these tests confirmed that in all cases, the single enzyme (*BamH1* and *Not1*) digestions produced linearized pRN3P plasmid, of the expected/theorised base-pair size, thus demonstrating the functionality of each restriction enzyme and suggesting a lack of digestion by one enzyme or the other was not the source of the failure to clone the insert [moreover, the literature from the manufacturer's of the restriction enzymes stated both enzymes should be compatible in a double digest with the used buffer (New England Biolabs 2019)]. Therefore, further experiments with different ligation conditions were conducted, where the ratio of vector and insert, as well as the overall concentration was varied. Although bacteria colonies grew relatively frequently after transformation of the ligation, they all proved to be false positives after screening for the inclusion of the EGFP-Tubulin-6 insert into the pRN3P multiple cloning site using colony PCR. It was not possible to ascertain why the cloning strategy failed and it could be explained by several reasons, including incomplete digestion by the restriction enzymes (van den Ent and Löwe 2006) or problems with the ligation conditions or ligase (although the same ligase had been successfully used by other lab members at the time).

4.1.2 TA-cloning into pGEM-T Easy vector

As described above the restriction enzyme digestion-based strategy to clone EGFP-Tubulin-6 insert into pRN3P proved unsuccessful. Therefore, an alternative approach to achieve the same aim of sub-cloning the PCR product derived insert, into a vector from which IVT could be employed to generate mRNA for embryo blastomere microinjection, was adopted; the so called 'TA-cloning' method. As described in the materials and methods section, the PCR derived insert was incubated with Taq DNA polymerase to catalyse the non-templated addition of one deoxyadenosine (A) to the 3' ends of each strand of the PCR product. This was then mixed with the commercially available vector, pGEM-T-Easy (that itself contains 3' deoxythymine/ T overhangs to provide a compatible end for introduction of the modified insert) and subject to DNA ligation (as described – and according to the manufacturer's protocols). In the experiment that resulted in a successful ligation, a concentration of 136.4 ng μL^{-1} for the insert and 45.2 ng μL^{-1} for the vector (*i.e.* an approximate 2.5:1 ratio of the insert to vector) was employed; of nine bacterial transformant colonies screened by colony PCR, two were found to contain the insert (shown in Figure 10)

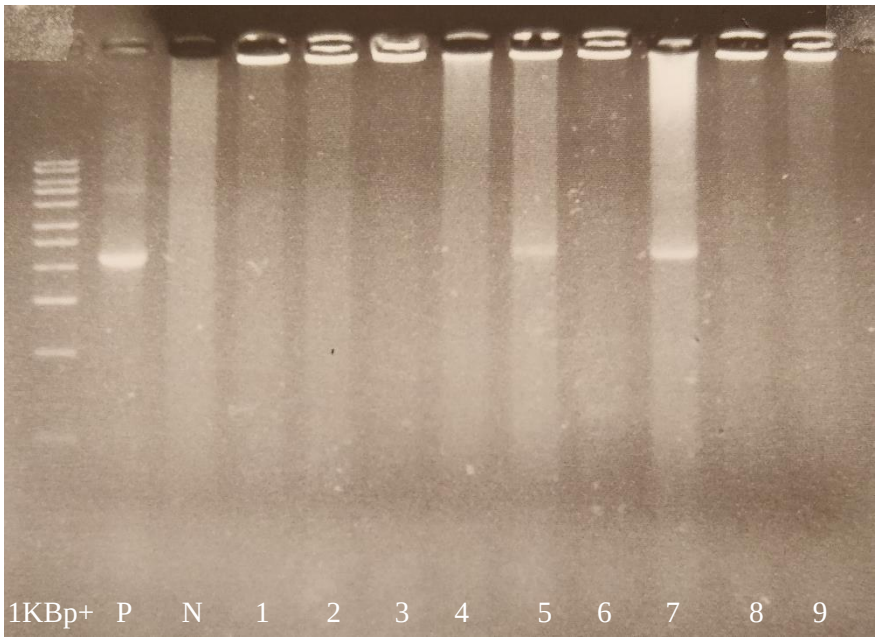

Figure 10 | **Confirmation of successful ligation of pEGFP-Tubulin-6 with pGEM-T Easy vector.** This agarose gel shows in the first column the DNA-marker 1KBp ladder, the second is loaded with a positive control for the insert and the third column a negative control with no plasmid loaded. The following columns depict the picked colonies after colony PCR. Bacterial colonies 5 and 7 show a band at the correct size of the insert and are thus confirmed to carry the ligated product.

The relative success of this method is explained by the superior effectiveness of the TA-cloning over the classic cohesive-end ligation, as it uses the property of Taq polymerase to add a single 3'-A overhang to both ends. This was aligned with the pGEM-T Easy vector, which has the complementary 3'-T overhang and can easily anneal to the insert (Zhou and Gomez-Sanchez 2000). However, whilst the successful cloning of the EGFP-Tubulin-6 into pGEM-T-Easy permits the possibility to generate mRNAs by IVT for embryo blastomere microinjection (using the T7 RNA polymerase promoter sequence in the pGEM-T-Easy vector), it lacks the advantage of cloning in pRN3P, as the stabilising 5' and 3' UTRs derived from the frog β-globin locus would not be incorporated into any derived transcripts.

4.1.3 Checking orientation of the EGFP-Tubulin-6 insert in the pGEM-T-Easy vector

Due to the nature of TA-cloning it is possible that the insert can successfully ligate in both head-on or tail-on orientations. However, only head-on orientations are compatible with IVT using the T7-RNA polymerase promoter sequence in pGEM-T-Easy (as this ensures the non-coding strand is transcribed into the correct sequence of codons in resulting mRNA). Therefore, to test the orientation of successfully cloned inserts in pGEM-T-Easy, two restriction enzymes, *BglII* and *NsiI*, were used to digest the plasmid. As *BglII* cuts off centre within the EGFP-Tubulin insert and *NsiI* cuts within the multiple cloning site of the pGEM-T-Easy vector, the size of the liberated fragment can be used to ascertain the orientation of the cloned insert. Accordingly, if the insert had been incorporated in the correct orientation, an expected liberated band size of 1432 base pairs would have been observable, whereas a band at 795 base pairs would denote an incorrectly oriented insert.

Figure 11 shows, that one of the two plasmid DNAs purified from the successfully transformed bacterial colonies had the EGFP-Tubulin-6 cloned in the correct orientation, whilst the other was cloned with the incorrect orientation. Therefore, a plasmid colony had been verified for IVT based mRNA production (after first being sequence verified).

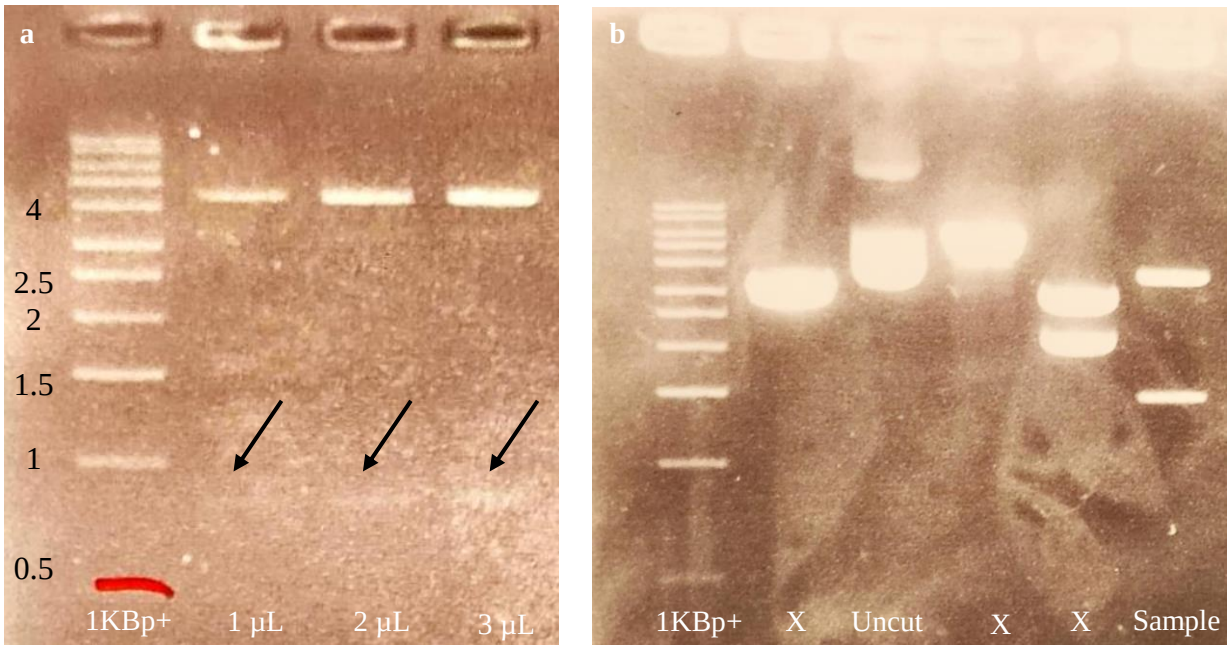

Figure 11 | **Control of the insert orientation.** By cutting the construct with the two chosen restriction enzymes (*BglII* and *NsiI*), the orientation of the insert was determined. 1KBp+ chosen as marker ladder (bottom to top [kb]: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10), with chosen marker sizes indicated **a.** Colony with wrong insert orientation, bands are visible at about 700 bp (indicated by arrows) compared to the ladder. Different amounts were loaded to check the intensity of the observed bands **b.** A colony with correct insert orientation on the last lane of this gel, a band is visible at around 1.5 kb, also the uncut pGEM-T-EGFP-Tubulin-6 construct is denoted on the third lane, lanes marked with X are to be disregarded, as these show different experiments.

4.1.4 Digestion of pGEM-T Easy construct and religation with pRN3P, plus *in-vitro* transcription (IVT)

The verified pGEM-T-EGFP-Tubulin6 construct was digested with *BamHI* and *NotI* to liberate the EGFP-Tubulin-6 insert, with the intention of trying to directly sub-clone the digest product into pRN3P (as it is a more desirable vector for IVT; as described above). The liberated insert and the vector were separated by agarose gel electrophoresis followed by gel extraction of an excised band containing the EGFP-Tubulin-6 insert. Several attempts were made to ligate the insert to pre-digested (*BamHI* and *NotI*) pRN3P, but ultimately proved unsuccessful. Therefore, it was decided for time constraint reasons to prepare the pGEM-T-Easy-EGFP-Tubulin-6 plasmid for IVT (by linearization) and mRNA was derived using the T7-RNA polymerase promoter. The derived mRNA was microinjected into mouse embryo blastomeres, by colleagues in the laboratory, but appeared not to be expressed as the desired EGFP-Tubulin-6 fusion protein (when inspected by confocal microscopy). It is possible this is because the derived IVT mRNAs transcripts were not stable enough in the *in vivo*

environment of the mouse blastomere. Therefore, alternative strategies to clone the EGFP-Tubulin-6 cDNA insert into pRN3P are under active consideration.

4.2 Embryo blastomere microinjection of HA-YAP(S112A) mRNA

To further study the effect of phosphorylation of Hippo-signalling related Yes-associated protein (YAP) during mouse preimplantation embryo development, a pre-existing construct pcDNA3.1-pA83:HA-YAP(S112A) was used in an IVT reaction to derive mRNA for embryo blastomere microinjection. HA-YAP(S112A) is a genetically modified variant of YAP in which a critical serine amino acid residue (that acts as a phosphorylation target for LATS1/2 kinases) is mutated to non-phosphorylatable alanine. It was anticipated expression of the mutated YAP in the developing preimplantation mouse embryo would potentiate TE specification, as activated hippo-signalling should not be able to cause its phosphorylation and sequestration to the cytosol in a manner that would be required for ICM specification. Therefore, the derived recombinant HA-YAP(S112A) mRNA was microinjected (by colleagues in the laboratory) into one blastomere of 2-cell stage mouse embryos (together with mRNA for histone H2A-RFP, to act as a marker of the microinjected clone and its subsequent progeny) that were then *in vitro* cultured to the 16- and 32-cell stages. Embryos were then fixed at these stages and the number of cells derived from the microinjected clone (or control non-microinjected clone) determined, as was their relative position within the embryo (*i.e.* outer or inner) determined by confocal microscopy (using DAPI as a DNA counter stain to visualise all nuclei within the embryo). Table 16-19 indicate the counting results and Figure 13 provides example micrographs of injected embryos.

Table 16 | YAP1(S112A) injected embryos in 16-cell stage and their cell localization.

16-cell stage embryos							
Embryo	Cell number	Injected cells	Non-injected cells	Injected outer cells	Non-injected outer cells	Injected inner cells	Non-injected inner dells
1	16	8	8	6	6	2	2
2	17	8	9	4	6	4	3
3	19	8	11	4	8	4	3
4	16	8	8	4	5	4	3
Average	17	8	9	4.5	6.25	3.5	2.75
Standard deviation	1.22	0.00	1.22	0.87	1.09	0.87	0.43
Two-tailed students t-test				p = 0.072		p = 0.228	

Table 17 | YAP1(S112A) injected embryos in transition from 16- to 32-cell stage and their cell localization.

16- to 32-cell stage embryos							
Embryo	Cell number	Injected cells	Non-injected cells	Injected outer cells	Non-injected outer cells	Injected inner cells	Non-injected inner dells
1	23	11	12	6	9	5	3
2	24	8	16	5	10	3	6
3	26	14	12	7	6	7	6
4	24	9	15	5	7	4	8
Average	24.25	10.5	13.75	5.75	8	4.75	5.75
Standard deviation	1.09	2.29	1.79	0.83	1.58	1.48	1.79
Two-tailed students t-test				p = 0.072		p = 0.483	

Table 18 | YAP1(S112A) injected embryos in 32-cell stage and their cell localization.

32-cell stage embryos							
Embryo	Cell number	Injected cells	Non-injected cells	Injected outer cells	Non-injected outer cells	Injected inner cells	Non-injected inner dells
1	32	16	16	12	10	4	6
2	30	14	16	11	10	3	6
3	32	15	17	10	10	5	7
4	32	16	16	9	11	7	5
5	32	16	16	8	10	8	6
6	30	14	16	8	10	6	6
7	31	16	15	10	10	6	5
8	33	16	17	8	10	8	7
9	27	11	16	5	9	6	7
10	32	16	16	9	9	7	7
Average	31.1	15	16.1	9	9.9	6	6.2
Standard deviation	1.64	1.55	0.54	1.84	0.54	1.55	0.75
Two-tailed students t-test				p = 0.177		p = 0.731	

Table 19 | YAP1(S112A) injected embryo in 64-cell stage and its cell localization.

64-cell stage embryo							
Embryo	Cell number	Injected cells	Non-injected cells	Injected outer cells	Non-injected outer cells	Injected inner cells	Non-injected inner dells
1	63	21	42	14	7	25	17

When assessing the data though, no significant difference in blastomere localization with respect to its outside or inside position could be observed between the non-microinjected cells and microinjected blastomeres. Microinjected blastomeres were expected to prefer the formation of the TE fate group. The assessed embryos are compared statistically in groups, defined by their developmental stage. This was conducted by two-tailed unpaired students t-tests, comparing the number of outside and inside cells between microinjected and non-microinjected blastomeres, the respective results are displayed in Table 16, 17 and 18. Figure 12 graphically represents the difference in commitment to the TE lineage on the outside of the compacted embryo. A single 64-cell stage embryo was assessed as well (Table 19) and whilst not being statistically robust at n = 1, it developed only 21 cells of the microinjected clone, but 42 non-injected blastomeres. These numbers surprise, as embryos in earlier development stages showed similar numbers of microinjected and non-injected clones, though more data is required to further study this observation.

The reason why we did not observe the predicted effect could reflect the fact that the recombinant protein is not being expressed or is expressed at a too low concentration. One way, that is planned, to test this is to perform immuno-fluorescence against the HA-epitope tag incorporated into the mRNA/fusion protein, after embryo microinjection of the recombinant mRNA. As this modified *YAP*-variant is not phosphorylatable at the specific targeted serine residue in this experiment, it was predicted it should accumulate in the nucleus of the cell (because it would not be retained in the cytosol by activated Hippo-signalling acting through the LATS1/2 kinases). And therefore activate the transcription factor TEAD4, which in turn transcriptionally activates TE fate specific genes, such as *Cdx2*. Such enhanced activation should be measurable. Therefore, to verify the functionality of the microinjected mRNA inside the blastomeres, a screening for TE fate specific protein expression like Cdx2 is proposed, as it has been done in previous studies (Nishioka et al. 2009). If the combined results of these experiments indicate that the recombinant YAP mRNA was expressed correctly, these results might also indicate that the type of division (symmetric or asymmetric) of the blastomeres is not dependent on the activity of YAP and thus not the TE fate proteins either. However, it is also possible that there is functional redundancy with other proteins that can assume the role of disrupted YAP (e.g. the related TAZ protein); although this seems unlikely if the non-phosphorylatable YAP was expressed at a suitable enhanced level; hence the expression level of the construct would be further matter for consideration

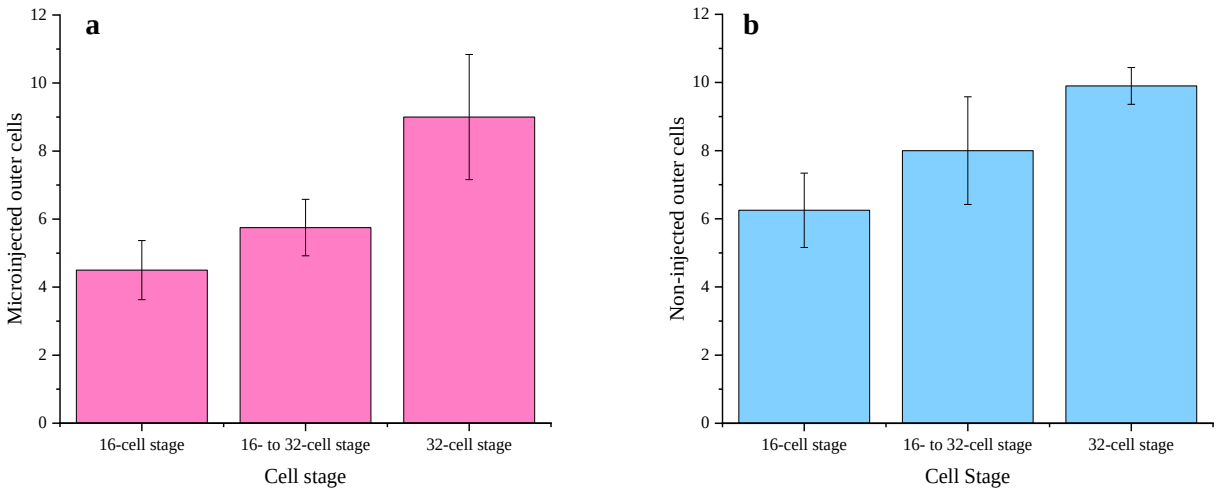

Figure 12 | **Comparison of outside microinjected and non-microinjected cells.** No significant difference in the commitment to the TE lineage at the outside of the compacted embryo was observed, when comparing injected and non-injected cells. Both graphs indicate the mean cell number at the individual developmental stage and the error bars denote the standard deviation. **a.** Microinjected outer cells. **b.** Non-injected outer cells

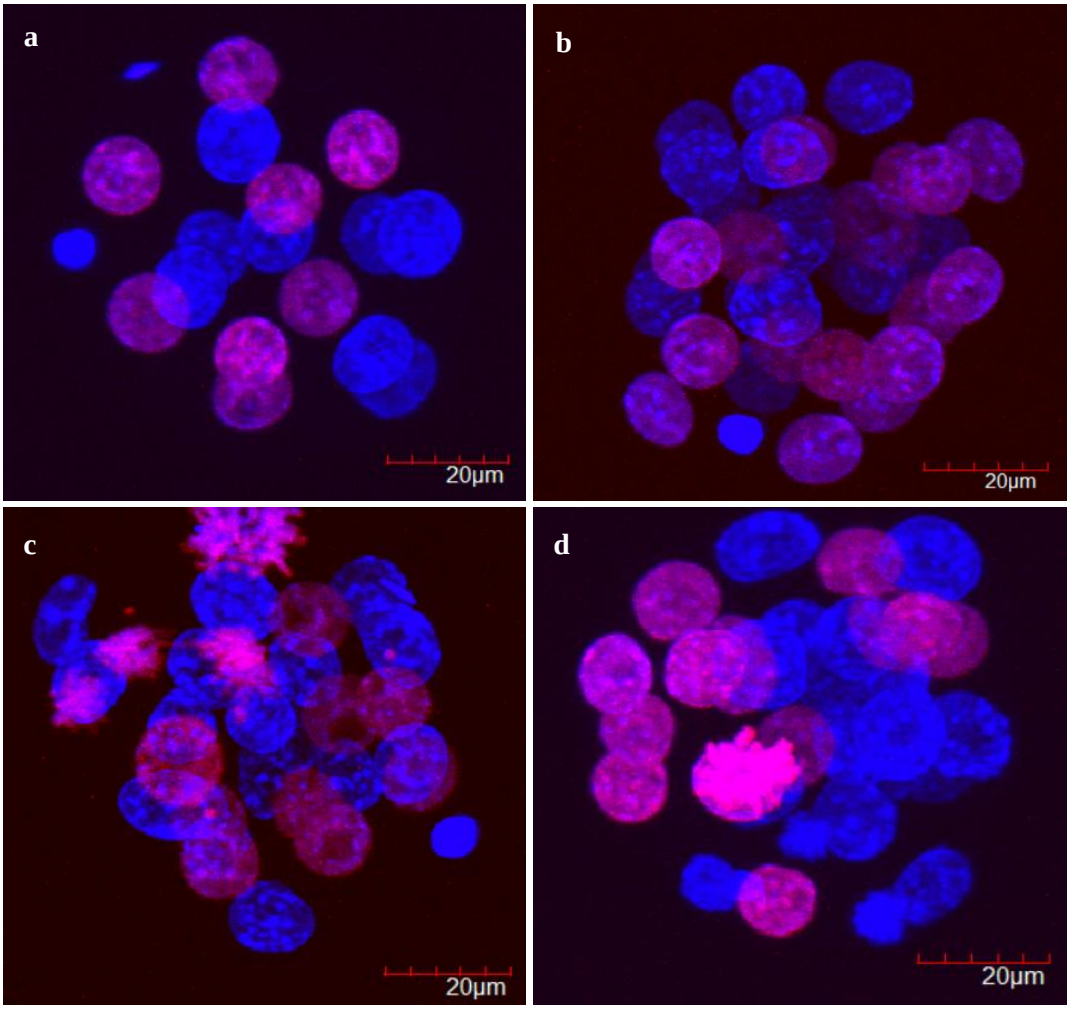

Figure 13 | **YAP(S112A) injected embryos.** Fluorescence labelled mouse embryos of Table 16 and 18 in a confocal microscope, in pink the injected blastomeres (H2aRFP) and in blue non-injected cells (DAPI). **a.** Embryo number 1 (16-cell stage embryos), **b.** Embryo number 1 (32-cell stage embryos), **c.** Embryo number 2 (32-cell stage embryos), **d.** Embryo number 3 (32-cell stage embryos).

4.3 Production (IVT) of Securin-GFP RNA

As a side project of this thesis, a recombinant Securin-GFP mRNA was produced using an IVT based protocol. Therefore, a pre-existing stock of pRN3P-securin-GFP plasmid containing bacterial clones was revived from -80 °C glycerol stock storage, plasmid purified, linearized and IVT (using the T3-RNA polymerase promoter sequence of the pRN3P vector) performed. Successful transcription was confirmed by gel electrophoresis (Figure 14). The derived recombinant Securin-GFP mRNA was then poly-adenylated to allow long term storage in -80 °C. Due to time restrictions, the mRNA was not injected into mouse embryos, although this is the intention of colleagues to study spindle assembly checkpoint system (SAC) (Musacchio and Salmon 2007).

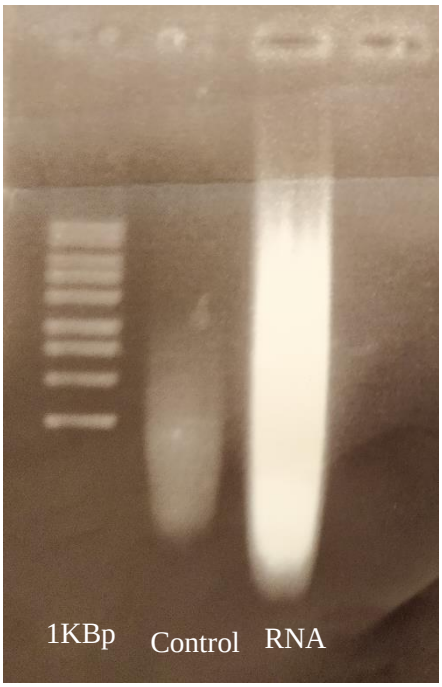

Figure 14 | **Transcription confirmation of securin-GFP.**

5 Conclusion

The main aim of this thesis was the successful ligation of EGFP-Tubulin-6 cDNA into pRN3P vector (ideally suited for the IVT of recombinant mRNA transcripts for microinjection and stabilised expression/ translation in mouse embryo blastomeres). Initial attempts to transfer the fusion protein cDNA from pEGFP-Tubulin-6 into pRN3P, by *BamHI* and *NotI* mediated restriction, proved unsuccessful. Variations/ optimisations to the reaction conditions and component concentrations did not change the result. After consulting different cloning methods, TA-cloning was chosen as an alternative. This method was successfully performed using a commercially provided kit, which involved utilising another cloning vector, pGEM-T-Easy, and yielded two bacterial transformants harbouring the desired insert (determined using colony PCR). Of two clones, the direction of the insert was determined/ verified, by applying restriction with the enzymes *BglII* and *NsiI*, and observing the resulting sizes of liberated fragments after gel electrophoresis; confirming one clone as having the EGFP-Tubulin-6 in the correct orientation (subsequently verified by Sanger DNA sequencing). However, it was not possible to use this clone as a source of insert for sub-cloning into the desired IVT vector pRN3P, hence this pGEM-T-Easy clone was used to derive EGFP-Tubulin-6 mRNA (by IVT) for mouse blastomere microinjection. Future, experiments should be conducted to ligate pEGFP-Tubulin6 into pRN3p with different ligation methods.

Transcription of YAP1(S112A) cDNA and consecutive microinjection into single 2-cell stage mouse embryo blastomeres was the second aim of this thesis. For the production of such recombinant mRNA, the pre-existing construct pcDNA3.1-pA83:HA-YAP1(S112A) was used in IVT. This variant of YAP1 was mutated at a site that does not allow for phosphorylation and cytoplasmic retention of the protein should not be possible, even under conditions of active Hippo-signalling and enhanced LATS1/2 kinase activity. The mRNA was microinjected into multiple embryos, together with fluorescent histone H2a-RFP mRNA to visualize the daughter cells of the injected blastomere. Using confocal microscopy, the embryos were imaged, cells counted and the respective position in the compacted embryos (from 16-cell stage onwards) recorded. Following the statistical data, there was no significant difference in the commitment of the microinjected cells observed, compared to non-microinjected blastomeres. Expression of *YAP* activated proteins, like *Cdx2* or *Gata3*, as well as *YAP* inhibited expression of *Sox2* or *Nanog* is to be conducted in future experiments.

Finally, as a side project, securin-GFP mRNA was produced by IVT transcription of the existing construct pRN3P-securin-GFP. Injection was not possible due to time constriction, this remains a subject of follow-up work.

6 Publication bibliography

Aiken, C. E. M.; Swoboda, P. P. L.; Skepper, J. N.; Johnson, M. H. (2004): The direct measurement of embryogenic volume and nucleo-cytoplasmic ratio during mouse pre-implantation development. In *Reproduction (Cambridge, England)* 128 (5), pp. 527–535. DOI: 10.1530/rep.1.00281.

Amon, A. (1999): The spindle checkpoint. In *Current Opinion in Genetics & Development* 9 (1), pp. 69–75. DOI: 10.1016/S0959-437X(99)80010-0.

Bayrhuber, H.; Hauber, W.; Kull, U. (2010): Linder Biologie. 23., neu bearb. Aufl. Hannover: Schroedel.

Chan, G. K.; Liu, S.-T.; Yen, T. J. (2005): Kinetochore structure and function. In *Trends in cell biology* 15 (11), pp. 589–598. DOI: 10.1016/j.tcb.2005.09.010.

Chazaud, C.; Yamanaka, Y. (2016): Lineage specification in the mouse preimplantation embryo. In *Development (Cambridge, England)* 143 (7), pp. 1063–1074. DOI: 10.1242/dev.128314.

Chazaud, C.; Yamanaka, Y.; Pawson, T.; Rossant, J. (2006): Early Lineage Segregation between Epiblast and Primitive Endoderm in Mouse Blastocysts through the Grb2-MAPK Pathway. In *Developmental cell* 10 (5), pp. 615–624. DOI: 10.1016/j.devcel.2006.02.020.

Desai, A.; Mitchison, T. J. (1997): Microtubule polymerization dynamics. In *Annual review of cell and developmental biology* 13, pp. 83–117. DOI: 10.1146/annurev.cellbio.13.1.83.

Dominguez-Brauer, C.; Thu, K. L.; Mason, J. M.; Blaser, H.; Bray, M. R.; Mak, T. W. (2015): Targeting Mitosis in Cancer: Emerging Strategies. In *Molecular Cell* 60 (4), pp. 524–536. DOI: 10.1016/j.molcel.2015.11.006.

Dong, J.; Feldmann, G.; Huang, J.; Wu, S.; Zhang, N.; Comerford, S. A. et al. (2007): Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. In *Cell* 130 (6), pp. 1120–1133. DOI: 10.1016/j.cell.2007.07.019.

Ducibella T.; Anderson E. (1975): Cell shape and membrane changes in the eight-cell mouse embryo: Prerequisites for morphogenesis of the blastocyst. In *Developmental Biology* 47 (1), pp. 45–58. DOI: 10.1016/0012-1606(75)90262-6.

Dziadek, M. (1979): Cell differentiation in isolated inner cell masses of mouse blastocysts in vitro: onset of specific gene expression. In *Journal of embryology and experimental morphology* 53, pp. 367–379.

Fleming T. P. (1987): A quantitative analysis of cell allocation to trophectoderm and inner cell mass in the mouse blastocyst. In *Developmental Biology* 119 (2), pp. 520–531. DOI: 10.1016/0012-1606(87)90055-8.

Hao, Y.; Chun, A.; Cheung, K.; Rashidi, B.; Yang, X. (2008): Tumor Suppressor LATS1 Is a Negative Regulator of Oncogene YAP. In *J. Biol. Chem.* 283 (9), pp. 5496–5509. DOI: 10.1074/jbc.M709037200.

Hauf, S.; Waizenegger, I. C.; Peters, J. M. (2001): Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. In *Science (New York, N.Y.)* 293 (5533), pp. 1320–1323. DOI: 10.1126/science.1061376.

Hirate, Y.; Hirahara, S.; Inoue, K.-i.; Suzuki, A.; Alarcon, V. B.; Akimoto, K. et al. (2013): Polarity-Dependent Distribution of Angiomotin Localizes Hippo Signaling in Preimplantation Embryos. In *Current Biology* 23 (13), pp. 1181–1194. DOI: 10.1016/j.cub.2013.05.014.

Hyafil, F. (1980): A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. In *Cell* 21 (3), pp. 927–934. DOI: 10.1016/0092-8674(80)90456-0.

Hyenne, V.; Louvet-Vallée, S.; El-Amraoui, A.; Petit, C.; Maro, B.; Simmler, M.-C. (2005): Vezatin, a protein associated to adherens junctions, is required for mouse blastocyst morphogenesis. In *Developmental Biology* 287 (1), pp. 180–191. DOI: 10.1016/j.ydbio.2005.09.004.

Jedrusik, A.; Parfitt, D.-E.; Guo, G.; Skamagki, M.; Grabarek, J. B.; Johnson, M. H. et al. (2008): Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. In *Genes & development* 22 (19), pp. 2692–2706. DOI: 10.1101/gad.486108.

Johnson, M. (1981): The foundation of two distinct cell lineages within the mouse morula. In *Cell* 24 (1), pp. 71–80. DOI: 10.1016/0092-8674(81)90502-X.

Johnson M. H.; McConnell M. L. (2004): Lineage allocation and cell polarity during mouse embryogenesis. In *Seminars in Cell & Developmental Biology* 15 (5), pp. 583–597. DOI: 10.1016/j.semcdb.2004.04.002.

Kelly, S. J. (1977): Studies of the developmental potential of 4- and 8-cell stage mouse blastomeres. In *The Journal of experimental zoology* 200 (3), pp. 365–376. DOI: 10.1002/jez.1402000307.

Kim, M.; Jho, E.-H. (2014): Cross-talk between Wnt/β-catenin and Hippo signaling pathways: a brief review. In *BMB reports* 47 (10), pp. 540–545. DOI: 10.5483/bmbrep.2014.47.10.177.

Mihajlović, A. I.; Bruce, A. W. (2016): Rho-associated protein kinase regulates subcellular localisation of Angiomotin and Hippo-signalling during preimplantation mouse embryo development. In *Reproductive BioMedicine Online* 33 (3), pp. 381–390. DOI: 10.1016/j.rbmo.2016.06.028.

Mihajlović, A-I.; Bruce, A. W. (2017): The first cell-fate decision of mouse preimplantation embryo development: integrating cell position and polarity. In *Open biology* 7 (11). DOI: 10.1098/rsob.170210.

Musacchio, A.; Hardwick, K. G. (2002): The spindle checkpoint: structural insights into dynamic signalling. In *Nature reviews. Molecular cell biology* 3 (10), pp. 731–741. DOI: 10.1038/nrm929.

Musacchio, A.; Salmon, E. D. (2007): The spindle-assembly checkpoint in space and time. In *Nature reviews. Molecular cell biology* 8 (5), pp. 379–393. DOI: 10.1038/nrm2163.

New england Biolabs (2019): BamHI, NotI: Restriction Enzyme Double Digestion. Available online at http://nebcloner.neb.com/#!/protocol/re/double/BamHI,NotI, checked on 11/21/2019.

Nishioka, N.; Inoue, K.-i.; Adachi, K.; Kiyonari, H.; Ota, M.; Ralston, A. et al. (2009): The Hippo Signaling Pathway Components Lats and Yap Pattern Tead4 Activity to Distinguish Mouse Trophectoderm from Inner Cell Mass. In *Developmental cell* 16 (3), pp. 398–410. DOI: 10.1016/j.devcel.2009.02.003.

Nogales, E.; Whittaker, M.; Milligan, R. A.; Downing, K. H. (1999): High-Resolution Model of the Microtubule. In *Cell* 96 (1), pp. 79–88. DOI: 10.1016/S0092-8674(00)80961-7.

O'Connor C. (2008): Cell Division: Stages of Mitosis. Nature Education 1(1):188.

Pan D. (2007): Hippo signaling in organ size control. In *Genes & development* 21 (8), pp. 886–897. DOI: 10.1101/gad.1536007.

Pan D. (2010): The hippo signaling pathway in development and cancer. In *Developmental cell* 19 (4), pp. 491–505. DOI: 10.1016/j.devcel.2010.09.011.

Peters, J.-M. (2006): The anaphase promoting complex/cyclosome: a machine designed to destroy. In *Nature reviews. Molecular cell biology* 7 (9), pp. 644–656. DOI: 10.1038/nrm1988.

Promega (2015): pGEM-T and pGEM-T Easy vector systems, Technical manual. Available online at https://at.promega.com/products/pcr/pcr-cloning/pgem-t-easy-vector-systems/?catNum=A1360#protocols, checked on 11/17/2019.

Saiz, N.; Plusa, B. (2013): Early cell fate decisions in the mouse embryo. In *Reproduction* 145 (3), R65-R80. DOI: 10.1530/REP-12-0381.

Sefton, M.; Johnson, M. H.; Clayton, L. (1992): Synthesis and phosphorylation of uvomorulin during mouse early development. In *Development (Cambridge, England)* 115 (1), pp. 313–318.

Sheth, B.; Nowak, R. L.; Anderson, R.; Kwong, W. Y.; Papenbrock, T.; Fleming, T. P. (2008): Tight junction protein ZO-2 expression and relative function of ZO-1 and ZO-2 during mouse blastocyst formation. In *Experimental Cell Research* 314 (18), pp. 3356–3368. DOI: 10.1016/j.yexcr.2008.08.021.

Straight, Aaron F. (1997): Cell cycle: Checkpoint proteins and kinetochores. In *Current Biology* 7 (10), R613-R616. DOI: 10.1016/S0960-9822(06)00315-0.

Strumpf, D.; Mao, C.-A.; Yamanaka, Y.; Ralston, A.; Chawengsaksophak, K.; Beck, F.; Rossant, J. (2005): Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. In *Development (Cambridge, England)* 132 (9), pp. 2093–2102. DOI: 10.1242/dev.01801.

van den Ent, F.; Löwe, J. (2006): RF cloning: a restriction-free method for inserting target genes into plasmids. In *Journal of biochemical and biophysical methods* 67 (1), pp. 67–74. DOI: 10.1016/j.jbbm.2005.12.008.

Villena, F. P.-M. de; Sapienza, C. (2001): Female Meiosis Drives Karyotypic Evolution in Mammals. In *Genetics* 159 (3), pp. 1179–1189. Available online at https://www.genetics.org/content/genetics/159/3/1179.full.pdf.

Wahl, P.; Paoletti, J.; Le Pecq, J. B. (1970): Decay of fluorescence emission anisotropy of the ethidium bromide-DNA complex. Evidence for an internal motion in DNA. In *Proceedings of the National Academy of Sciences of the United States of America* 65 (2), pp. 417–421. DOI: 10.1073/pnas.65.2.417.

Walczak, C. E.; Heald, R. (2008): Mechanisms of Mitotic Spindle Assembly and Function. In Kwang W. Jeon (Ed.): International review of cytology. A survey of cell biology, vol. 265. London: Academic Press (International Review of Cytology), pp. 111–158.

Watson, A. J. (1992): The cell biology of blastocyst development. In *Molecular reproduction and development* 33 (4), pp. 492–504. DOI: 10.1002/mrd.1080330417.

Watson, A. J.; Kidder, G. M. (1988): Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. In *Developmental Biology* 126 (1), pp. 80–90. DOI: 10.1016/0012-1606(88)90241-2.

Wiley, L. M.; Eglitis, M. A. (1981): Cell surface and cytoskeletal elements: Cavitation in the mouse preimplantation embryo. In *Developmental Biology* 86 (2), pp. 493–501. DOI: 10.1016/0012-1606(81)90207-4.

Yagi, R.; Chen, L.-F.; Shigesada, K.; Murakami, Y.; Ito, Y. (1999): A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator. In *The EMBO Journal* 18 (9), pp. 2551–2562. DOI: 10.1093/emboj/18.9.2551.

Yagi, R.; Kohn, M. J.; Karavanova, I.; Kaneko, K. J.; Vullhorst, D.; DePamphilis, M. L.; Buonanno, A. (2007): Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. In *Development (Cambridge, England)* 134 (21), pp. 3827–3836. DOI: 10.1242/dev.010223.

Yamanaka, Y.; Ralston, A.; Stephenson, R. O.; Rossant, J. (2006): Cell and molecular regulation of the mouse blastocyst. In *Developmental dynamics : an official publication of the American Association of Anatomists* 235 (9), pp. 2301–2314. DOI: 10.1002/dvdy.20844.

Zernicka-Goetz, M.; Morris, S. A.; Bruce, A. W. (2009): Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. In *Nat Rev Genet* 10 (7), pp. 467–477. DOI: 10.1038/nrg2564.

Zhou, M.-Y.; Gomez-Sanchez, C. E. (2000): Universal TA Cloning. In *Curr. Issues Mol. Biol.* (2), pp. 1–7.

7 Appendix

pEGFP-Tubulin-6 DNA Sequence

ATGGTGAGC - EGFP Sequence (717 Nucleotides - 239 Amino Acids)
ACTAACTAG - Tubulin Sequence (1353 Nucleotides - 451 Amino Acids: Missing ATG Start)
LINKER - 6 Amino Acids (18 Nucleotides) between EGFP and Tubulin
ATG - Methionine Start Codon
TAG TAA TGA - Stop Codons
AGATCT – BglII restriction site

AAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGG
TCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGTCGCCACCATGGT
GAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAA
ACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
AAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTAC
GGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATG
CCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGC
CGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGG
AGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATG
GCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAG
CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGA
CAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGG
TCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTCCGGAC
TCAGATCTCGAGTGCGTGAGTGCATCTCCATCCACGTTGGCCAGGCTGGTGTCCAGATTGGCAATG
CCTGCTGGGAGCTCTACTGCCTGGAACACGGCATCCAGCCCGATGGCCAGATGCCAAGTGACAAG
ACCATTGGGGGAGGAGATGACTCCTTCAACACCTTCTTCAGTGAGACGGGCGCTGGCAAGCACGTG
CCCCGGGCTGTGTTTGTAGACTTGGAACCCACAGTCATTGATGAAGTTCGCACTGGCACCTACCGC
CAGCTCTTCCACCCTGAGCAGCTCATCACAGGCAAGGAAGATGCTGCCAATAACTATGCCCGAGGG
CACTACACCATTGGCAAGGAGATCATTGACCTTGTGTTGGACCGAATTCGCAAGCTGGCTGACCAG
TGCACCGGTCTTCAGGGCTTCTTGGTTTTCCACAGCTTTGGTGGGGGAACTGGTTCTGGGTTCACCT
CCCTGCTCATGGAACGTCTCTCAGTTGATTATGGCAAGAAGTCCAAGCTGGAGTTCTCCATTTACCC
AGCACCCCAGGTTTCCACAGCTGTAGTTGAGCCCTACAACTCCATCCTCACCACCCACACCACCCT
GGAGCACTCTGATTGTGCCTTCATGGTAGACAATGAGGCCATCTATGACATCTGTCGTAGAAACCT
CGATATCGAGCGCCCAACCTACACTAACCTTAACCGCCTTATTAGCCAGATTGTGTCCTCCATCACT
GCTTCCCTGAGATTTGATGGAGCCCTGAATGTTGACCTGACAGAATTCCAGACCAACCTGGTGCCC
TACCCCCGCATCCACTTCCCTCTGGCCACATATGCCCCTGTCATCTCTGCTGAGAAAGCCTACCATG
AACAGCTTTCTGTAGCAGAGATCACCAATGCTTGCTTTGAGCCAGCCAACCAGATGGTGAAATGTG
ACCCTCGCCATGGTAAATACATGGCTTGCTGCCTGTTGTACCGTGGTGACGTGGTTCCCAAAGATGT
CAATGCTGCCATTGCCACCATCAAAACCAAGCGCAGCATCCAGTTTGTGGATTGGTGCCCCACTGG
CTTCAAGGTTGGCATCAACTACCAGCCTCCCACTGTGGTGCCTGGTGGAGACCTGGCCAAGGTACA
GAGAGCTGTGTGCATGCTGAGCAACACCACAGCCATTGCTGAGGCCTGGGCTCGCCTGGACCACAA
GTTTGACCTGATGTATGCCAAGCGTGCCTTTGTTCACTGGTACGTGGGTGAGGGGATGGAGGAAGG
CGAGTTTTCAGAGGCCCGTGAAGATATGGCTGCCCTTGAGAAGGATTATGAGGAGGTTGGTGTGGA
TTCTGTTGAAGGAGAGGGTGAGGAAGAAGGAGAGGAATACTAAGGATCCACCGGATCTAGATAAC
TGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCC
CCTGAACC