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Functional Analysis of CHES1 During Mouse Development

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FUNCTIONAL ANAYLSIS OF *CHES1*
DURING MOUSE DEVELOPMENT

A Senior Honors Project
In Partial Fulfillment of
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ABSTRACT

The purpose of this project was to investigate the role of the Checkpoint Suppressor 1 (*Ches1*) gene in mammalian development. The *Ches1* gene belongs to the fork head transcription factor family and has been implicated in controlling the G2-M phase of the cell cycle in lower eukaryotes. Furthermore, Ches1 has been shown to bind the Sin3/Rpd3 HDAC complex in an inhibitory manner in yeast. In human tumor cell lines, Ches1 appears to negatively regulate gene expression through the recruitment of SKIP. While the functional role of Ches1 (Foxn3) in higher mammalian models is not well understood, it is under-expressed in both renal cell and oral squamous cell carcinomas.

Our initial analysis indicates that we have generated Ches1 mutant mice, and we plan to characterize the mutant mice for phenotypes that relate to developmental disorders as well as cancer formation. Our preliminary data suggests that loss of *Ches1* results in embryonic lethality in mice. Our studies on the expression pattern of *Ches1* in 11.5 days post coitum embryos show marked expression of Ches1 throughout the spinal cord. Observations of our Ches1 mutant colony have resulted in the phenotypic characterization of distinct skeletal abnormalities in *Ches1* heterozygotes. Future experiments will test our deduction of embryonic lethality, will further characterize *Ches1* expression in embryonic development, and will address cancer susceptibility.

THE CELL CYCLE

The cell cycle consists of four phases with the ultimate goal of replicating the genetic information encoded on the DNA and then segregating it equally into two daughter cells. The four phases are gap 1 (G1), synthesis (S), gap 2 (G2), and mitosis (M).

Cells enter G1 after completion of a previous cycle. During this phase of variable length, the cell grows and proceeds through its normal metabolic functions. The cell subsequently proceeds to S phase, in which the DNA is replicated. Each chromosome replicates semi-conservatively to form two identical sister chromatids. After replication, sister chromatids remain attached to one another at the centromere via cohesin protein complexes. The kinetochore, a trilaminar structure composed of many proteins, is also formed at the centromere and functions to link the chromatids to spindle fibers later during mitosis. After S phase, the cell proceeds to G2, in which cell growth occurs in anticipation of division.

Mitosis, or cell division, generates two daughter nuclei that are identical to the parent nucleus. Mitosis is divided into five continuous stages: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, the chromosomes condense, the two centrosomes move to opposite poles of the cell, and the mitotic spindle is formed as microtubules are synthesized from each centrosome. Prometaphase begins as the nuclear envelope disintegrates, which allows microtubules of the spindle to attach to the kinetochore of each chromatid. Each sister chromatid of one chromosome must attach to opposite poles for

proper orientation. During metaphase, the chromosomes become aligned at a midpoint known as the metaphase plate of the cell. After proper alignment and kinetochore attachment is achieved, anaphase occurs. During anaphase, the sister chromatids separate and move toward opposite poles of the cell. In telophase, nuclear envelopes reform around each of the two clusters of daughter chromosomes. Finally, cell division is completed by cytokinesis, the process that divides the cytoplasm into two distinct daughter cells, each containing a nucleus with identical chromosomes.

Coordination of the stages and intricate processes in the cell cycle is driven by kinase complexes. The active form of these complexes consists of at least two proteins, the catalytic subunit known as a cyclin-dependent-kinase (CDK) and the regulatory subunit known as a cyclin (Hartwell and Kastan, 1994). These complexes undergo changes in the CDK and cyclin subunits that drive the cycle from one stage to another. In *Saccharomyces cerevisiae*, a single kinase, CDC28, interacts successively with a series of transiently expressed cyclins (Nasmyth, 1993). Each cyclin has a periodic spike in expression to drive a phase of the cell cycle, and the cyclin protein is rapidly degraded after translation (Molinari, 2000). The situation in mammalian cells is considerably more complicated as multiple CDK's and cyclins function to regulate cell cycle progression; however, the overall theme remains the same.

CELL CYCLE CHECKPOINTS

Cell cycle checkpoints are mechanisms that ensure the order of events in the cell cycle and that integrate DNA repair with cell cycle progression (Hartwell and Kastan, 1994). These checkpoints make sure that progression to the next event occurs only after completion of a prior event in the cell cycle. If certain conditions have not been met or if DNA damage is present, the cycle will arrest at these checkpoints.

There are four major areas of cell cycle control: the G1/S checkpoint, the intra-S phase checkpoint, the G2/M checkpoint, and the spindle checkpoint before anaphase in mitosis (Molinari, 2000). The G1/S checkpoint prevents the cell from replicating its DNA if any damage is present (Hartwell and Kastan, 1994). In mammalian cells in G1, the dominant checkpoint response to DNA damage is the ATM(ATR)/CHK2(CHK1)–p53/MDM2-p21 pathway, which is capable of inducing sustained G1 arrest (Kastan and Lim, 2000). An arrest of the cell cycle may allow time for DNA repair or may commit a damaged cell to apoptosis (Weinert, 1998).

The intra-S phase checkpoint is activated by genotoxic stress in the cell during replication. In the presence of such stress, the firing of origins of replication is inhibited in order to slow DNA synthesis (Kastan and Bartek, 2004). There are two parallel pathways to this checkpoint, both of which are controlled through ATM/ATR signaling (Kastan and Bartek, 2004).

The G2/M checkpoint prevents progression into mitosis in the presence of either DNA damage or incompletely replicated DNA (Hartwell and Kastan, 1994).

In *S. cerevisiae*, the RAD9, RAD17, RAD24, MEC1 (a functional homologue of the human ATM gene), MEC2, and MEC3 gene products block mitosis in the presence of DNA damage or if replication is blocked in late S phase; MEC1 and MEC2 also prevent mitosis if replication is blocked in early S phase (Hartwell and Kastan, 1994). This pathway is not yet well understood in mammals.

The spindle checkpoint ensures the integrity of segregation of sister chromatids in mitosis. This checkpoint prevents the onset of anaphase until the following prerequisites are achieved: assembly of a bipolar spindle, attachment of kinetochores of sister chromatids to spindle fibers emanating from opposite poles, and arrival of the attached chromosomes at the metaphase plate (Elledge, 1996). While many of the details of this pathway are not yet understood, it is thought that a lack of tension at the kinetochore and/or an unattached kinetochore activates the checkpoint response (Amon, 1999).

CHECKPOINT SUPPRESSOR 1

CHES1 (Checkpoint suppressor 1; *FOXN3*), a novel human cDNA, was first isolated as a high-copy suppressor of the *S. cerevisiae* G2/M checkpoint mutants *rad9, mec1, rad24, rad53,* and *dun1* (Pati et al., 1997). *CHES1* encodes a 490 amino acid member of the forkhead/winged helix family and has been mapped to a region between 14q24.3 and 14q31 (Pati et al., 1997).

The forkhead family is a large family of transcription factors that share a structurally related DNA binding domain: the forkhead. This domain is approximately 110 amino acids long, and it folds into a structure with three alpha

helices and three beta strands, which assemble into a compact hydrophobic core
(Granadino et al., 2000). Members of this family have been found in a wide
range of species, with the exception of green plants (Granadino et al., 2000).
Forkhead proteins bind DNA as monomers and regulate transcription
independently, either as activators or repressors. In some cases, however, they
can also serve as transcriptionally inert docking factors for other proteins with
transcriptional regulatory domains (Granadino et al., 2000).

CHES1 belongs to the FOXN subfamily of forkhead transcription factors.
This subfamily contains five members in addition to CHES1: FOXN1 (WHN), a
regulator of keratinocyte growth and differentiation of thymic epithelium (Coffer
and Burgering, 2004); FOXN2 (human T-cell leukemia virus enhancer factor),
which binds to the human T-cell virus long terminal repeat and may be involved
in transcriptional regulation (Li et al., 1992); and FOXN4-6, which have only
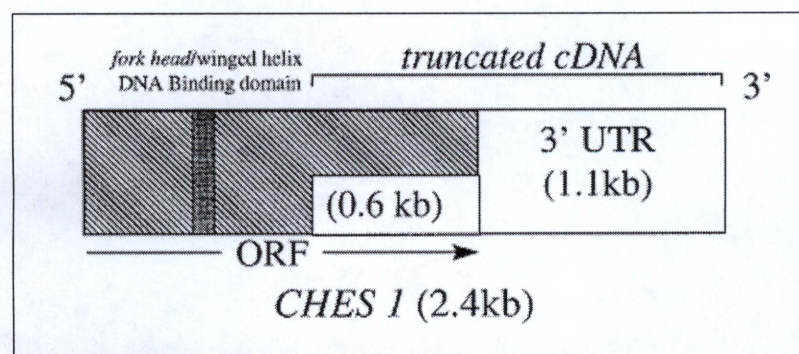recently been identified (Katoh and Katoh, 2004a-c).

Figure 1. Schematic diagram of the full-length CHES1 cDNA (Pati et al., 1997)

The CHES1 clone first isolated by the work of Pati et al. only encoded the
carboxy terminus of the full protein, which lacks the forkhead DNA binding

domain (Fig. 1). This truncated cDNA conferred increased survival of the

aforementioned G2/M checkpoint mutants after exposure to UV irradiation,

ionizing irradiation, and methylmethane sulfonate (MMS) (Pati et al., 1997).

Furthermore, this suppression was accompanied by a reconstitution of a wild-

type G2 arrest after DNA damage in spite of mutations in essential checkpoint

genes (Pati et al., 1997). *CHES1* was also able to suppress the null alleles of

MEC1--which has been described as being essential for growth in the absence of

DNA damage (Kato and Ogawa, 1994)—*RAD9,* and *DUN1* (Pati et al., 1997).

Because the forkhead domain was not included in the truncated *CHES1* clone

used in these experiments, it does not appear that Ches1 induces new genes for

restoration of the checkpoint. Pati et al. proposed that Ches1 may activate an

alternative *MEC1*-independent pathway which results in G2 arrest after damage
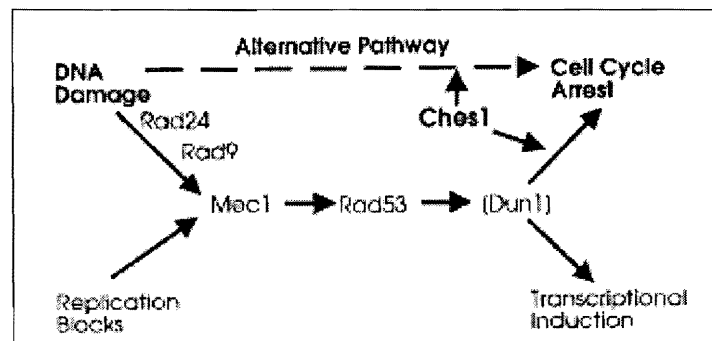
(Fig. 2).

Figure 2. Possible mechanism of Ches1 action in cell cycle control (Pati et al., 1997)

In a study aimed at further elucidating information on the Ches1

mechanism in yeast, the C-terminus of Ches1 was fused to glutathione *S*-

transferase (GST) in order to identify *S. cerevisiae* proteins that interact with the

Ches1 *in vivo* (Scott and Plon, 2003). It was found that the Ches1 fusion protein

interacts with Sin3, a component of the Sin3/Rpd3 histone deacetylase complex
(HDAC) in budding yeast (Scott and Plon, 2003). Rather than binding directly to
DNA, the Sin3/Rpd3 HDAC complex is targeted to specific promoter regions via
Sin3 interactions with site-specific DNA-binding proteins (Kadosh and Struhl,
1997).

The HDAC complex is a type of chromatin remodeling complex that
removes acetyl groups on lysine residues of the amino-terminal ends of histones,
particularly histones H3 and H4. The modulation of chromatin is a major route of
gene regulation. Specifically, acetyl groups post-translationally added to these
lysine residues neutralize the positive charge of the amino acid, which diminishes
the electrostatic interaction of the histone with the negatively-charged DNA,
thereby loosening chromatin structure (Neely and Workman, 2002). This
loosening of chromatin structure by acetylation makes the DNA more accessible
to transcriptional machinery for gene expression. Therefore, deacetylation via
HDACs accomplishes the opposite: it restores the positive charge of the lysine
residue to allow a stronger and tighter interaction between histone and DNA,
effectively repressing transcription.

In the study of Ches1 and Sin3 interaction, Ches1 did not suppress the
DNA damage response in *sin3* mutants, and over-expression of *SIN3* blocked
the Ches1-mediated G2 arrest after DNA damage (Scott and Plon, 2003). This
evidence implies that the Ches1 mechanism in suppressing *S. cerevisiae*
checkpoint mutants and in restoring G2 arrest functions through the inhibition of
Sin3/Rpd3 HDAC activity. By inhibiting this complex, target lysine residues

would remain acetylated and target gene expression would continue. This conclusion is consistent with prior work, which demonstrated that acetylation of H3 and H4 histone tails is necessary for the cell cycle transition of G2 to M phase in the absence of DNA damage (Howe et al., 2001). Further investigation into the mechanism of DNA damage-induced arrest in both *sin3* and *rpd3* mutants revealed the dependency of the reconstituted G2/M arrest on the *MAD1*-dependent spindle checkpoint pathway (Scott and Plon, 2003).

Scott and Plon published a second study in 2005 on Ches1 function in human cells. In this study, the C-terminus of Ches1 was shown to consistently repress transcription when targeted to a reporter promoter in cell lines derived from tumor tissues. Through screening of a cDNA library derived from fetal brain tissue and subsequent co-immunoprecipitation assays, Ches1 was found to interact with SKIP (Ski –interacting protein) (Scott and Plon, 2005). SKIP is a well-conserved transcriptional adaptor protein that functions to recruit either activation or repression complexes to mediate multiple signaling pathways involved in the control of cell proliferation and differentiation (Dahl et al., 1998). Specifically, Ches1 directly interacts with the hydrophobic C-terminus of SKIP (aa 470-536), which defines a new region of SKIP protein-protein interaction (Scott and Plon, 2005). The findings in this study imply that Ches1 negatively regulates transcription through recruitment of SKIP. SKIP has been shown to bind the following repression complex members: mSin3a, HDAC1, and HDAC2 (Laduron et al., 2004). This data is consistent with the finding that Ches1 inhibits the yeast orthologs of these proteins (Scott and Plon, 2003).

CHES1 AND CANCER

In light of Ches1's involvement in suppressing checkpoint mutant phenotypes and in restoring G2 arrest after DNA damage in *S. cerevisiae*, we hypothesize that loss of Ches1 function will lead to higher cancer incidence in mammalian cells. The G2/M checkpoint functions to arrest the cell cycle in the presence of DNA damage or unreplicated DNA before entering mitosis. Loss of this control could allow a cell to enter mitosis with damage and pass its mutations on to its daughter cell(s). Incorporation of mutations can lead to loss of protein function and genetic instability, each of which make the cell more susceptible to further damage. Mutations of cell cycle checkpoint genes increase genetic instability and accelerate the process of cellular evolution toward the loss of cell cycle control, the hallmark of cancer (Hartwell and Kastan, 1994).

Recent studies have shown *CHES1* to be under-expressed in multiple tumor types. Struckmann et al. reported a 60% *CHES1* expression frequency in normal renal tissue with only a 14% expression frequency in clear cell renal cell carcinomas (cRCC). *CHES1* was also under-expressed in samples of oral squamous cell carcinoma (OSCC) as compared to normal tissues (Chang et al., 2005). Specifically, it was under-expressed in 46% of tumor samples studied, and, on average, its expression was decreased by 15.03-fold in OSCC cells (Chang et al., 2005). Chang et al. also noted a correlation between the under-expression of *CHES1* and the expression of *CDK1* in oral cancer samples, which they interpreted as an indication of a relationship between the regulatory mechanisms of the two genes. The down-regulation of *CHES1* in malignant

tissues along with the finding that Ches1 recruits SKIP to repress transcription

indicate that genes regulated by Ches1 may be over-expressed in cancerous

cells due to decreased Ches1 levels (Scott and Plon, 2005).

RESULTS AND METHODS

Generation of Ches1 Mouse Models

In order to study the effects of a loss-of-function mutation of *CHES1* in

mammals, *Ches1* deficient mice were generated by the following means. Murine

embryonic stem (ES) cells with a gene-trap inserted into the *Ches1* gene were

obtained from the Mutant Mouse Regional Resource Center (MMRRC, UC-

Davis). A schematic of the *Ches1* gene in *Mus musculus* is shown in Figure 3

and a schematic of the gene-trap insertion is shown in Figure 4. The gene-trap

has a strong splice acceptor site that disrupts the normal splicing of *CHES1*

mRNA, which results in a non-functional, truncated Ches1 protein. The location

of the trap was found to be downstream of exon 2 according to the automated 5'-

RACE annotation provided by the MMRRC.

These embryonic stem cells were used for blastocyst injections, and the

resulting embryos were implanted into pseudo-pregnant females. The result of

these transgenic embryos was the generation of chimeric mice. A chimera has

some cells derived from the host's ES cells while other cells are derived from the

transgenic ES cells (129 Ola/Hsd background), which in this case are the cells

with *Ches1* mutated. The chimeras were mated to c57/BL6 wild type female

mice to ascertain whether the *Ches1* mutant cells contributed to the germ line of

the chimeras. If they had, some of the resulting offspring would be heterozygous

for *Ches1* and would have an agouti coat color. Offspring of the chimera and

wild type crosses were genotyped using PCR (polymerase chain reaction)

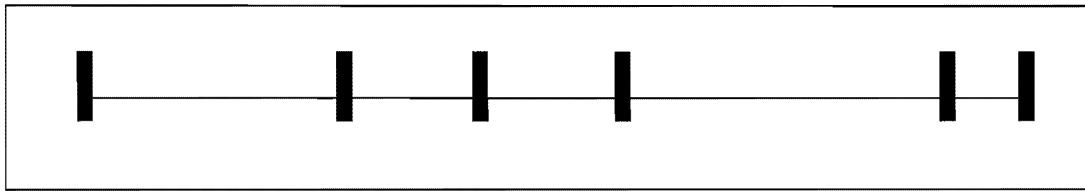analysis, and heterozygous mice were bred to expand the colony.

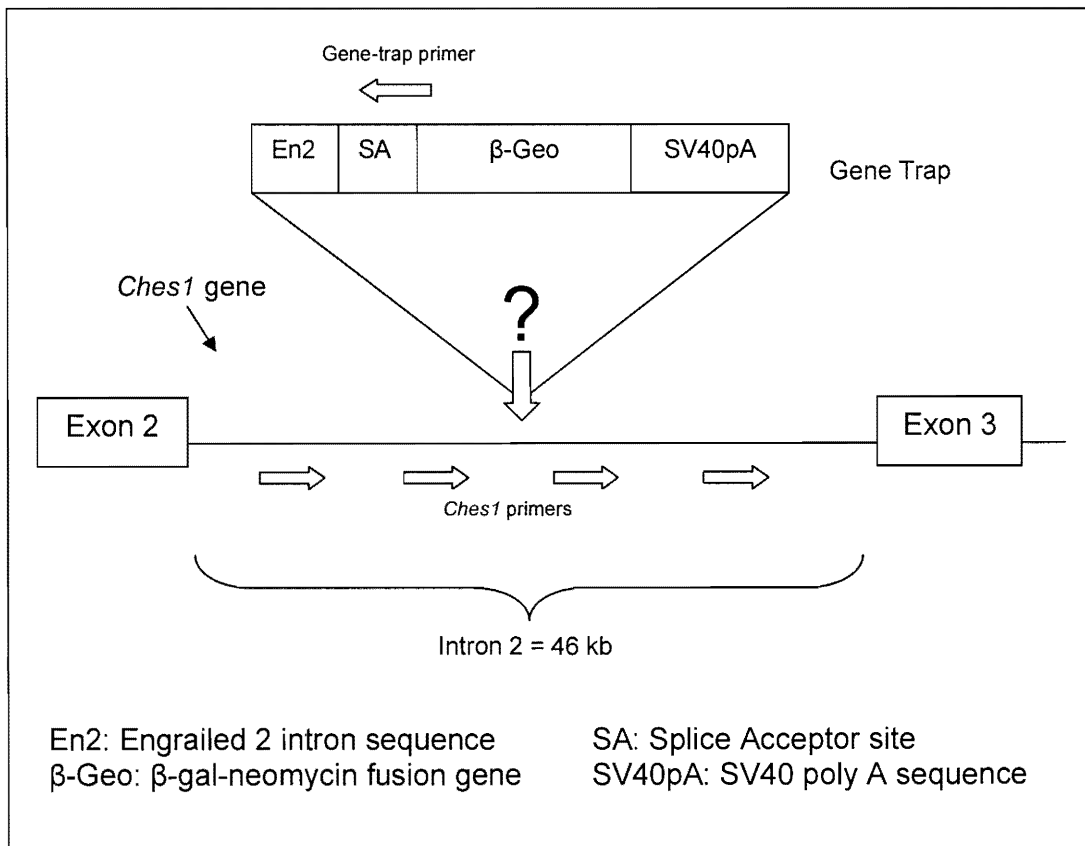Figure 3. *Ches1* Gene in *Mus musculus* . Exons 1-6 shown from left to right as black rectangles.

Figure 4. Schematic representation of the gene-trap disruption of *Ches1*.

PCR Analyses and Genotyping

Despite the 5'RACE confirmation of the location of the gene-trap provided by the Mutant Mouse Regional Resource Center, PCR analysis utilizing gene specific primers and trap specific primers have failed. We designed primers specific for intron 2 and the gene-trap to amplify a *Ches1*-gene-trap product that is expected in the heterozygous offspring. Analysis with at least a dozen different primer pairs spanning the whole of intron 2 did not provide any mutant specific PCR product. If the trap is inserted into the 46 kb-long intron 2, this problem may be due to the enormous size of the intron and/or the redundancy of intron sequences within the mouse genome. Furthermore, our experiments are based on a mouse genome database that is not completely curated.

Consequently, our efforts in genotyping offspring of the founder mice have been limited to PCR amplification of gene-trap sequences using two gene-trap primers. While this analysis enables us to confirm the presence of the gene-trap within a mouse's genome, it does not enable confirmation of the location of the gene-trap within the *Ches1* gene. Furthermore, gene-trap specific PCR analysis does not enable differentiation between *Ches1* heterozygous and nullizygous mice.

5'RACE: Rapid Amplification of cDNA Ends

Due to unsuccessful efforts to design a PCR that will amplify a Ches1-gene-trap product and due to a 1% chance of clonal contamination, we employed 5'RACE (5' <u>R</u>apid <u>A</u>mplification of <u>c</u>DNA <u>E</u>nds) to confirm the presence of the

gene-trap in the *Ches1* gene (Figure 5). Total RNA was isolated from the spleen

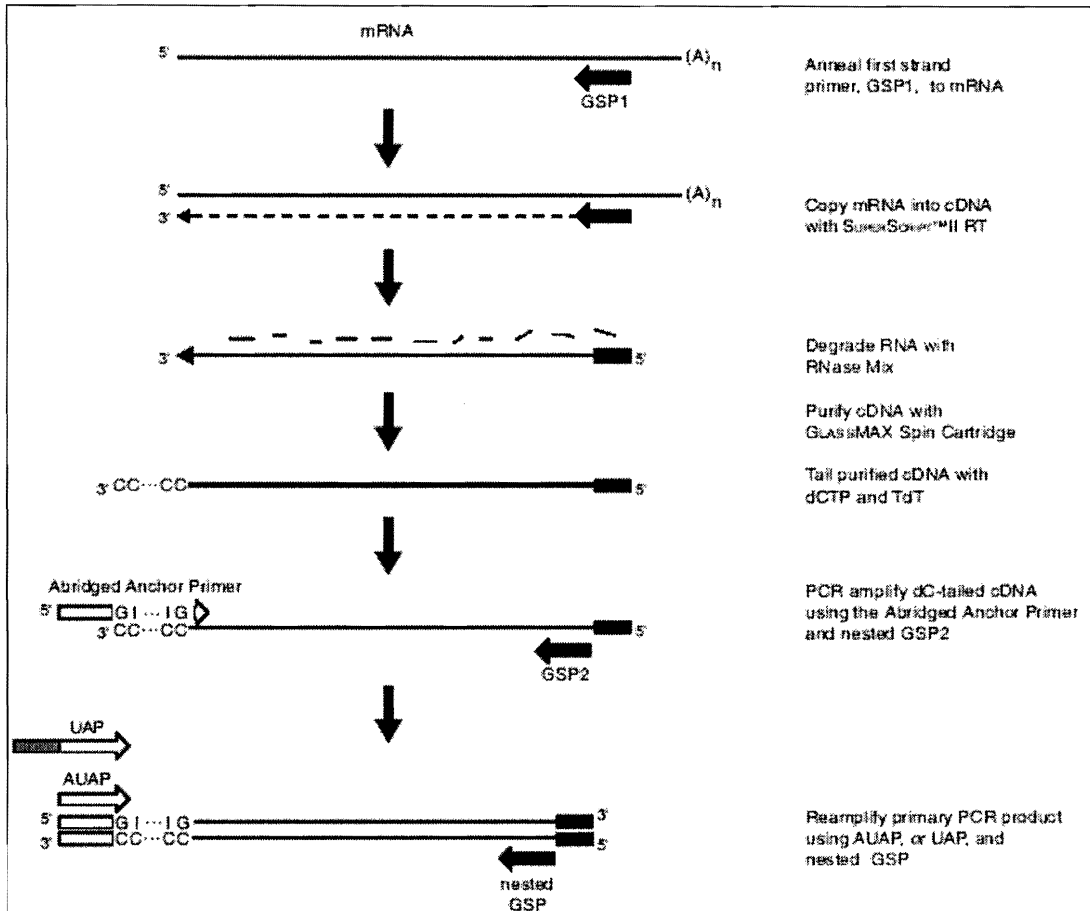of a *Ches1* heterozygous mouse using TRIzol reagent (Invitrogen).

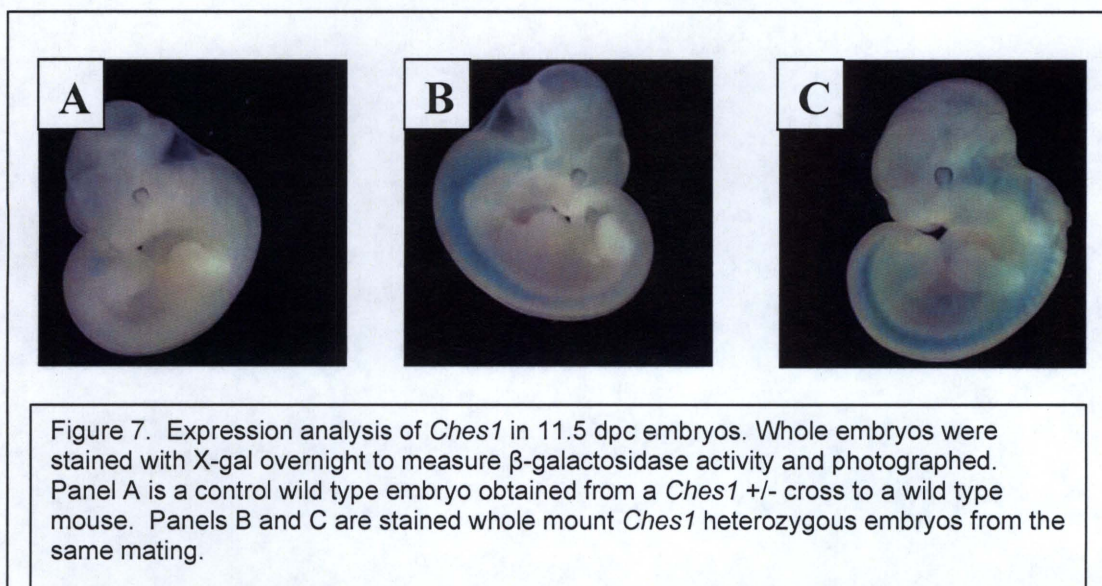Figure 5. Overview of the 5'RACE Procedure (Invitrogen).

Following isolation of total RNA, a reverse transcriptase reaction was

carried out using a trap specific primer to synthesize cDNA from the trap locus to

the 5' end of the transcript it's located on. After purification of the product, a

homopolymeric tailing reaction was completed using terminal deoxynucleotidyl

transferase (TdT) to add dC's to the 3'end of the newly synthesized cDNA. The

dC tail creates a binding site for primers necessary to amplify the cDNA product.

Two subsequent PCR amplification reactions were carried out using primer pairs

consisting of a trap primer and an anchor primer followed by agarose gel

electrophoresis. Our analysis of the agarose gel electrophoresis of the 5'RACE

reaction shows an 850 bp product (Fig. 6). The next step in confirming the

location of the trap is to purify and

sequence this identified fragment.

Figure 6. PCR amplification of 5'RACE product.
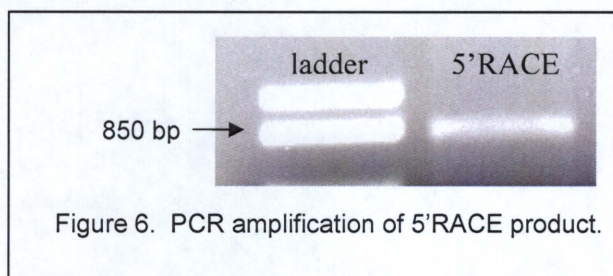
Ches1 Expression in Development and Ches1 Phenotypes

To investigate possible roles and expression patterns for *Ches1* in mouse

development, we used X-gal and the promoter-less β-galactosidase-neomycin

fusion construct (Fig. 4) to determine gene expression patterns in an 11.5 days

post coitum (dpc) mating of a heterozygotic male to a wild type female (Fig. 7).

Tissues with blue staining indicate expression of β-Galactosidase from the gene

Figure 7. Expression analysis of *Ches1* in 11.5 dpc embryos. Whole embryos were stained with X-gal overnight to measure β-galactosidase activity and photographed. Panel A is a control wild type embryo obtained from a *Ches1* +/- cross to a wild type mouse. Panels B and C are stained whole mount *Ches1* heterozygous embryos from the same mating.

trap, which is driven by the Ches1 promoter. As shown in Figures 7B and 7C, *Ches1* expression was evident in the spinal cord of the heterozygous embryos. In phenotypic characterization of adult mice, our observations of *Ches1* heterozygotes have revealed distinct skeletal abnormalities.

Analysis of Heterozygous Intercrosses

Because of unsuccessful efforts to develop a PCR that will amplify a Ches1-gene-trap product, we have been unable to genotypically differentiate between *Ches1* heterozygous and nullizygous mice. Consequently, we can not definitively confirm whether the *Ches1* nullizygous condition is embryonic lethal or not. However, through analysis of heterozygous intercrosses and the resulting offspring, the actual number of heterozygous/nullizygous offspring appears conspicuously low as compared to the expected number based on a Mendelian distribution (Table 1). This suggests embryonic lethality of the *Ches1* nullizygous condition. Furthermore, our data shows that the average number of pups per litter of a wild type mating is between seven and eight. Based on *Ches1* data from seven heterozygous intercrosses, the average number of pups per litter is approximately five. This further supports our conclusion that *Ches1* is necessary for embryonic development.

Table 1. Heterozygous intercross results. Genomic DNA samples obtained from tail clippings were analyzed by PCR to determine their *Ches1* genotype.

Results of Heterozygous Intercrosses		
	Wild Type	Het/Null
Actual Number	15	21
Expected Number*	15	45
*Based on a Mendelian distribution		

Generation of Ches1 Deficient Cell Lines

Mouse embryonic fibroblasts (MEFs) were obtained from a timed mating between a heterozygous male and a heterozygous female. Briefly, two 13.5 dpc embryos were harvested from the pregnant female and disaggregated using a syringe. The embryonic tissues were then plated onto 100 mm tissue culture plates and passaged upon confluency. These cell lines were maintained at 37°C using humidified air supplemented with 5% CO_2 in DMEM with 15% fetal bovine serum and Penstrep. PCR amplification of a trap sequence using yolk sac samples genotyped both embryos as heterozygous (or possibly nullizygous) for *Ches1*. The cell line generated from the first embryo was later found to be contaminated. The second cell line was frozen in passage two until null and wild type cell lines are generated for comparison of cell growth kinetics.

Future Work

Further research on murine *Ches1*-deficient models is needed to expand our current understanding of *Ches1's* role in mammalian development and the cell cycle. To advance the data presented in this paper, we plan to examine embryonic expression data for *Ches1* at 7.5, 9.5, 13.5 and 15.5 dpc. This will allow for a more comprehensive perspective of the gene's role in development. To confirm the presence and locus of the gene-trap in *Ches1*, we will proceed with our 5'RACE analysis by sequencing the 850 bp product. Once data on the location of the trap is obtained, a PCR to amplify a *Ches1*-gene-trap product will be designed in order to differentiate between heterozygotes and nullizygotes.

This PCR will allow for the retro-analysis of DNA samples from our *Ches1* colony to determine whether any nullizygous mice have been born of heterozygous intercrosses. If the results of said genotyping support our initial deduction that loss of *Ches1* leads to embryonic lethality, we plan to determine the day and mechanism of embryonic lethality. Additionally, we intend to generate wild type and null cell lines in order to do cell growth kinetic experiments and cell cycle analyses for comparisons between the three genotypes. Upon *Ches1* colony expansion, we will investigate organismal cancer susceptibility and relative life span.

ACKNOWLEDGEMENTS

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