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PRINCIPAL INVESTIGATOR: Roel Nusse, Ph.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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Roel Nusse, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Stanford University
Stanford, California 94305-5401

email musse@cmgm.stanford.edu

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13. Abstract ***(Maximum 200 Words)*** (abstract should contain no proprietary or confidential information)

This grant was aimed at identifying tumor suppressor genes, one of the major goals of contemporary cancer research. A promising new approach in these efforts is to take advantage of the fact that growth regulatory genes can be easily identified in the fruit fly *Drosophila* and that such genes are highly conserved in evolution. We have found a novel gene in *Drosophila*, *tartaruga* (*tar*) that is involved in repressing target genes of an important signaling pathway, the Wnt pathway. The *tar* gene is highly conserved and this grant addresses whether the mammalian homologs of *tar* are involved in cancer. We have characterized a mouse homolog of *tar* at the molecular level. We also found a second homolog of the gene in mouse and human DNA. We have generated mice with constitutive or conditional-loss-of-function mutations in the gene. We demonstrated that the *tar* gene is essential for normal development. We have also mapped the human *tar* gene(s) on the genome.

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INTRODUCTION

The goal of this grant has been to clone, map, and characterize the mammalian *tartaruga (tar)* gene in order to understand more about its function in cancer and, secondarily, in development. Our approach in the mouse (project 1) has been to study expression at the RNA level, and to map the genomic structure in order to generate a knock out mouse for examination of the loss of function phenotype. Once the mouse is derived, it will be exposed to carcinogenic agents in order to uncover tumor susceptibility. We cloned both mouse homologs of *tartaruga* and established the genomic organization of these genes. We built replacement vectors for both genes. In the last year of the grant, we have successfully inactivated *tartaruga 2* in the mouse germ line. The gene is essential for mouse development, as the mutant embryos die during gestation. This is an important advance because it demonstrates that these genes control many developmental events.

Our approach in the human (project 2) was to study the expression pattern using Northern blot analysis and, after mapping the gene, to examine the locus for linkage to known LOH regions. This work has been completed.

BODY

Project 1: The mouse *tar* genes

Work done in year 1: Expression of the mouse *tar* gene at RNA level

To examine the expression of mouse *tar* at the RNA level, multiple tissue Northern blots from adult tissues as well as developmental Northern blots from whole embryos at days 7,11,15 and 17 were made or obtained commercially. The blots were examined with cDNA probes made from IMAGE clones identified in a BLAST search with the conserved regions of *Drosophila tartaruga.* Additionally, frozen sections of adult organs were examined by in situ hybridization using riboprobes made from the same clones. We learned that mouse *tartaruga* is expressed at the earliest day examined, that it is expressed in all tissues examined, and that it produces three transcripts that are 8.5, 6.5, and 6.0 kb in length. After cloning the gene(below), we used RT-PCR to examine RNA from a variety of tissues in order to discover transcript specificities.

Work done in year 1: Clone full-length *tar* cDNA

In order to clone the full length cDNA, we screened a day 14 embryonic cDNA library in which we knew *tartaruga* was highly expressed, based on the work above. The cDNA clones obtained contained a great deal of genomic DNA, but after sequencing over 40 clones, we were able to assemble a 3 kb cDNA contig for which every splice junction was confirmed by at least one spliced clone. We found that the open reading frame, which coded for a 615 aa protein, had alternate 3' ends after the most 3' conserved region

ended, explaining two of the three transcripts observed. We did not find the 3' poly A signal, so it is possible that alternate 3' UTR lengths explain the third transcript. We then probed the Northern blots with distinct regions of the ORF, but did not identify any more differences in the three transcripts. Importantly, we found that the 5' conserved leucine zipper was present in all three transcripts

Work done in year 2: clone full-length second *tar* gene and study expression

When examining human cDNA clones, we discovered that there was a second mammalian homolog of *tartaruga*. Because the two human paralogs were highly conserved, it seemed likely that both were functional, and that there probably was a mouse paralog also. Because a second mouse *tartaruga* could obscure the phenotype of the first, we realized we would have to clone the second gene if it existed and knock it out also, even though we were now into the second year of the project.

By searching the GenBank mouse EST databases with short pieces of the 5' region of the human paralog, we were able to find an IMAGE clone containing a short (24 nucleotides) homologous region; however, the clone itself had been lost by the supplier. Using the sequence, we designed a primer that allowed us to amplify the intervening diagnostic zinc finger region by RT-PCR from mouse RNA, establishing that the second gene existed. We were then able to locate ESTs based on the extensive (2kb) 3' UTR , eventually finding an IMAGE clone long enough to contain half of the ORF. By alternately using human and mouse sequences to search the EST and genomic databases we were able to assemble a 4.2kb contig and have a cDNA clone that covers most of this region. Our expression studies of the second tartaruga were less extensive, consisting of Northern blot analysis only. As with *tartaruga 1*, there were three transcripts. In this case, there were no alternate 3' ends, but there were 2 different poly A signals.

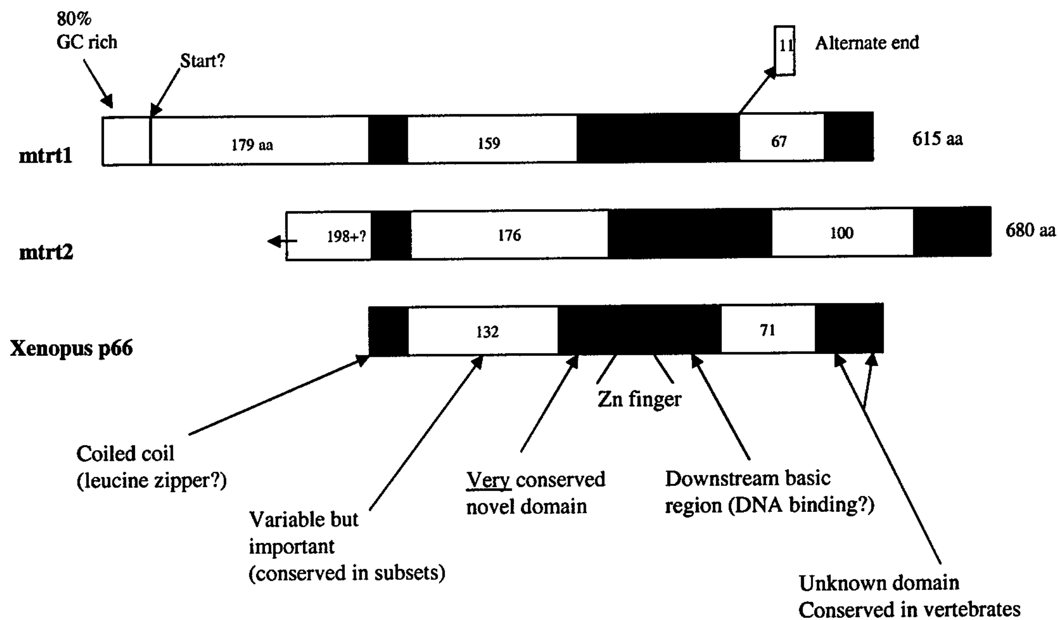
We screened the BAC library and obtained 5 positive clones, then mapped the gene structure as with *tartaruga 1*. Before we were through, however, the mouse genomic sequence (C57BL strain) for this gene starting 3kb 5' of what we believe is the second exon was deposited in Genbank. We have confirmed the published structure and relevant restriction sites with our BACs, which come from strain 129, the source strain for the ES cells we are using.

Work done in year 2:Generate *tar* knockout constructs

The *tartaruga* proteins have two highly conserved regions, a 5' leucine zipper and a 3' zinc finger with very conserved flanking arms. We have extensively mapped the genomic DNA of both genes by restriction enzymes and have designed assays for recognition of correctly recombined ES cells. We have developed a cloning strategy in which the 3' and 5' arms were cut from the BACs and placed into a replacement vector containing *pgk thymidine kinase* and *pgk neo* selection markers The *pgkneo* marker is flanked by loxP sites which can be removed by transient Cre-recombinase expression after homologous recombination at the ES cell level. The strategy was designed so that with minor

modifications, the reagents being generated could also be used to build conditional knock-out constructs.

Knockout strategy:

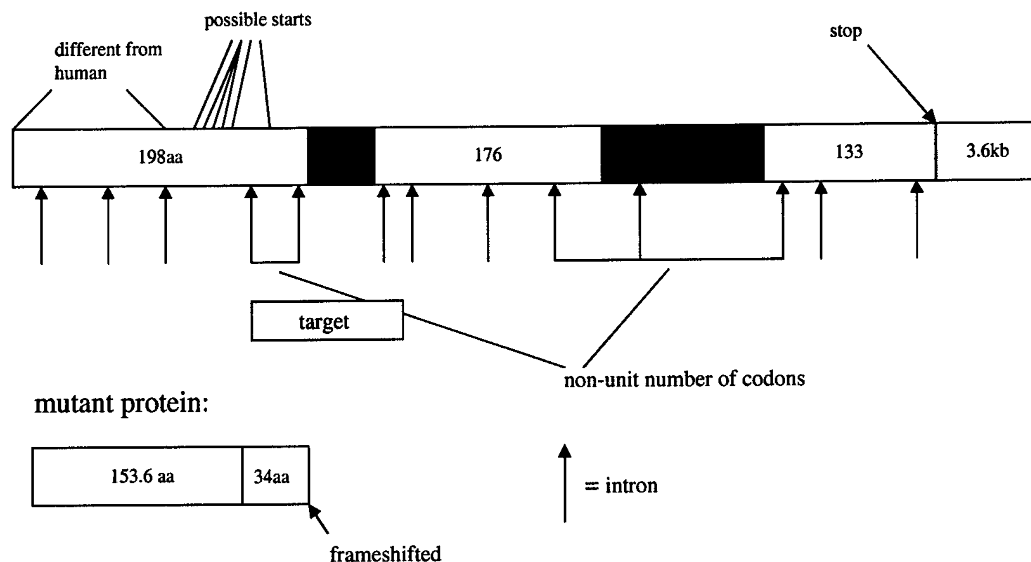


Tartaruga protein domains: mtrt1: mouse *tartaruga 1*; **mtrt2**: mouse *tartaruga 2* or mouse *p66;* **Xenopus p66** is the frog homolog of **mtrt2**, identified as a member of the NuRD or Mi-2 histone deacetylase complex (Wade *et al.*,Nature Genetics 23:62).

The leucine zipper is present in all three transcripts of both genes. In each gene, the exon prior to the leucine zipper contains a non-unit number of codons. If this exon and the following exon are removed, the leucine zipper is removed and a frame shift that cannot be corrected by any combination of downstream exons is introduced. This strategy was used in designing constructs for both *tar2* (also called *trt2* or *p66*) and *tar1* (also called *trt1*).

Generation of chimeras for *tartaruga 2*

In order to utililize appropriate restriction sites for building the construct, we decided to remove 3 exons in the target region of *tartaruga 2*.



***Tartaruga 2/p66* mutation strategy:** A replacement vector was used to substitute a floxed *pgkneo* cassette for the three-exon target region, removing the leucine zipper and introducing an uncorrectable frameshift.

1.3 kb of DNA 5' of the three-exon target region and 6.3 kb of DNA 3' of the target region were removed from 129SV BACs to serve as homologous arms. These were inserted on either side of a floxed *pgkneo* cassette in the ploxPNT plasmid, obtained from Janet Rossant at University of Toronto. Distal to the short arm was a *pgk thymidine kinase* cassette, and a *Not 1* linearization site was at the distal end of the long arm.

To assess recombination, we developed both long-range PCR and Southern blot assays. For the PCR assay we designed primers from non-repetitive regions of DNA exterior to the homologous arm to be paired with primers from the *pgkneo* cassette, for both arms. The short arm Southern assay relied on a 464nt difference between the location of an *Nco1* site in the original target region compared to its location in the *pgkneo* cassette which would replace the target region in a successful recombination. The 559nt probe for this assay was made by PCR from a non-repetitive region exterior to the short arm.
The long arm Southern assay was difficult to design because most restriction enzymes had sites within the 6.3kb region. However, a combination of *Sac1* and *Spe1* produced a 1091 nt difference due to the disappearance of a *Sac1* site in the target region and the addition of a *Spe1* site in the *pgkneo* cassette. The 537 nt probe was made by PCR from a non-repetitive region exterior to the long arm. The templates for both Southern blot probes were129 SV BACs.

Work done in Years 2-3

We obtained R1 and Tc1 mouse embryonic stem cells and optimized growing conditions in our laboratory. We then electroporated the *tartaruga 2* construct into the ES cells and selected with G418 and gancyclovir. After three electroporations, we obtained 48 independent clones that survived the selection. Our PCR assay revealed that 4 clones had integrated correctly over the short arm, however we could not confirm that correct integration had occurred over the long arm by PCR, and also had numerous additional ambiguous results with the short arm PCR assay. Therefore we amplified all 48 clones in order to produce enough DNA for Southern blot analysis, and confirmed correct integration of both arms in the original 4 clones using this assay(see figures above). The Stanford transgenic facility then injected these clones into C57BL blastocysts that were subsequently implanted into pseudopregnant mothers. These injections have produced 12 chimeras from 3 clones of the R1 cell line, and 2 chimeras from the Tc1 clone. Of the existing chimeras, 11 exhibit high percentages (60-100%) of chimerism, as assessed by coat color. At maturity these animals were crossed with C57BL mates in order to determine germline transmission of the ES cell line and mutation by coat color and tail biopsy, respectively.

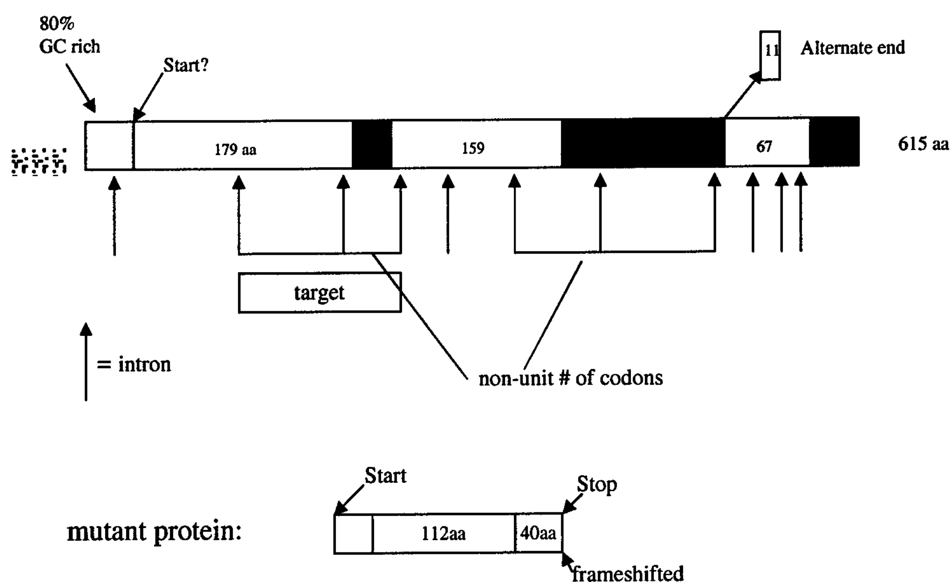
From this analysis, several chimeric animals originally derived from separate clones appeared to have mutant cells in the germ line. From these mice, we obtained offspring animals that were heterozygous for the *tartaruga 2* mutation, as generated originally by the homologous recombination. By PCR and Southern blot analysis, we confirmed the heterozygosity of these animals.

This was an important step, as germ line transmission of ES cells manipulated by homologous recombination and targeted mutagenesis is of the critical event in these knock-out strategies. It also allowed us to test for a possible requirement of the *tartaruga 2* gene, by intercrossing heterozygous animals. The offspring animals were genotyped by PCR and Southern blotting, to examine how the mutant gene was transmitted.
In 95 offspring mice, we detected 29 animals that were homozygous wild type, 66 heterozygous animals and none that were homozygous mutant. We concluded that the *tartaruga 2* gene is essential for normal development, as no viable homozygous mutant animals were obtained. Otherwise, there was a normal Mendelian ratio in the offspring, indicating that loss of a single allele of this gene has no consequences.

Further analysis indicated that the homozugous mutant mice died in utero. The embryos could be genotyped by PCR on extra-embryonic tissue. The mutant mice stop developing around day 7.5, and have several defects. We are currently in the process of analyzing these defects in more detail.

Attempted generation of chimeras for *tartaruga 1*

In order to utilize appropriate restriction sites for removal of homologous DNA from the BACs, we decided to use a two exon target region and to insert the arms in the reverse orientation relative to the *pgkneo* and *pgk thymidine kinase* cassettes.



***Tartaruga 1* mutation strategy**: A replacement vector is being used to substitute a *pgkneo* cassette for the two-exon target region, removing the leucine zipper and introducing a frameshift that cannot be corrected by alternate splicing.

5.7 kb of DNA 5' of the two-exon target region and 2.5 kb of DNA 3' of the target region were removed from 129SV BACs to serve as homologous arms. These were inserted into the plox PNT plasmid described above, with the linearization site at the distal end of the long arm and the *pgk thymidine kinase* cassette distal to the short arm.

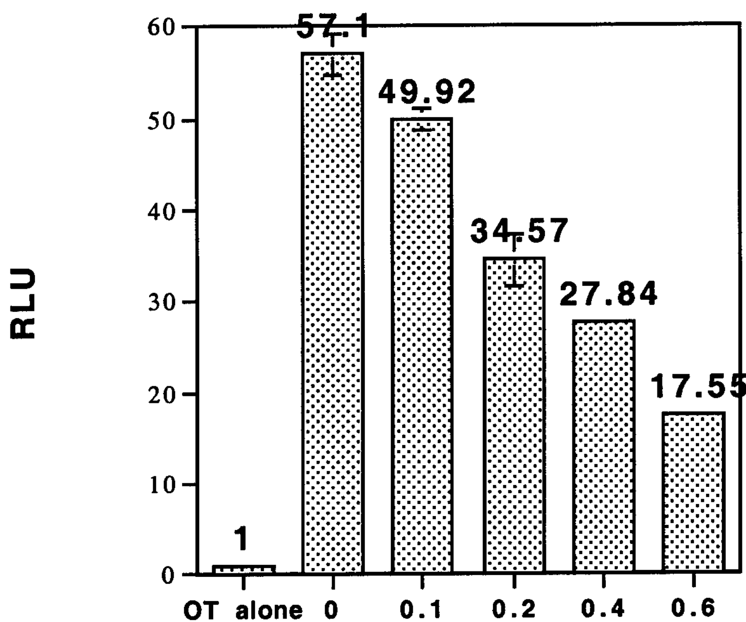
To assess recombination we developed both long range PCR and Southern blot assays as described above for *tartaruga 2.* Both long and short arms can be assessed on *BamH1* digests, due to the introduction of a *BamH1* site in the inserted region. Probes exterior to each homologous arm detect a 13.1 kb band in wild type DNA, but a 6.1 kb band over the short arm and a 7.18 band over the long arm in the mutant. Alternate Southern analyses can be carried out with *Sca1* and *Nco1* digests if needed.

Although we have electroporated the *tartaruga 1* construct into both R1 and Tc1 cells a combined total of five times, we have not yet obtained any homologous recombinants. We have obtained 700 clones so far that have survived selection. Of these, 699 appear to be negative by both PCR and Southern blot assays. The one remaining clone could however not be confirmed by Southern blotting.

trt inhibits β–catenin activation of a TCF reporter

Work in Drosophila suggested tartaruga may function as a negative regulator of Wnt signaling. To further investigate tartaruga's role as a repressor of Wnt signaling, we

examined its effects on the activation of the TCF-responsive OT reporter. The OT plasmid contains three copies of a TCF-binding motif upstream of a minimal c-fos promoter. We transfected 293 cells with the OT reporter and β-catenin. As expected, β-catenin efficiently activated the OT reporter. Co-expression of tartaruga inhibited β–catenin activation of the OT reporter in a dose-dependent manner, maximally inhibiting activation by 70%.



OT (μg)	0.1	0.1	0.1	0.1	0.1	0.1
βgal (μg)	0.1	0.1	0.1	0.1	0.1	0.1
β-catenin (μg)	0	0.2	0.2	0.2	0.2	0.2
trt (μg)	0	0	0.1	0.2	0.4	0.6
GFP (μg)	0.8	0.6	0.5	0.4	0.2	0

The effects of tartaruga on β–catenin activation of the OT reporter were examined in 293 cells. The OT plasmid was co-transfected with β–catenin and increasing amounts of tartaruga. Luciferase activity was measured 48 hours post-transfection. All transfections also included a plasmid encoding βgal (EF-1α βgal) to normalize for transfection efficiency by measurement of β-galactosidase activity. Measurements were perfomed in duplicate.

Project 2, the human *tar* gene
Work done in year 1: Study expression pattern

Probes made from human cDNA IMAGE clones were used to examine human multiple tissue Northern blots. As with mouse *tartaruga 1,* there were three widely

expressed transcripts of lengths almost identical to those of the mouse. The second *tartaruga* gene was also found to produce three transcripts.

Work done in year 1: Map human tar genes on chromosomes

When looking for mouse *tartaruga* homologs using Southern blot analysis, we discovered a *Bgl II* polymorphism between the *C57BL/6J* and *M. spretus* strains. We used this polymorphism to genotype the Jackson Laboratory Backcross DNA mapping DNA panels, thus mapping *tartaruga 1* to within 0.5 cM of the Npr1 locus of Chromosome 3. By synteny, we had mapped the human *tartaruga* to human Chromosome 1, band q21-q22.

In order to map *tartaruga 2*, we searched the genome databases with additional sequence we had obtained by sequencing *tartaruga 2* IMAGE clones and identified a sequence tagged site that had been mapped four times. *Tartaruga 2* was thus mapped to Chromosome 19, interval D195899-D195407.

We did not find obvious candidate tumor suppressor genes or linkage with LOH regions at either of these loci. This line of research has not been pursued since.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning mouse homologs of tartaruga
- Establishing genomic organization genes
- Building targeting constructs and generating chimeric mice with mutant cells.
- Demonstrating that tartaruga 2 is essential for normal development, as germ line mutations in the gene lead to embryonic lethality.
- Establishing that tartaruga is a repressor of gene expression

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have accomplished the major goals of the grant. During year one, we have made significant progress in our aim of generating loss of function mutations in the mouse tartaruga gene. We have also characterized a second mouse gene and two human homologs. Importantly, during the past year we have accomplished the most technically demanding step: generating mutant ES cells in which tartaruga is targeted, and deriving chimeric offspring mice. We also have found direct evidence that tartaruga is a repressor of gene expression. Finally, and most importantly, we have obtained germ line transmission of the mutant allele of tartaruga 2, and demonstrated that the gene is essential for normal development.

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APPENDICES

None