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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

E-Mail: rnusse@cmgm.stanford.edu

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13. ABSTRACT ***(Maximum 200 Words)***

This grant is aimed at identifying tumor suppressor genes, one of the major goals of contemporary cancer research. 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 obtained direct evidence that tar can act as a repressor of gene expression. 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 are in the process of generating mice with constitutive or conditional-loss-of function mutations in the gene. Constructs for these gene targeting experiments have been transfected and chimeric mice have been obtained. We have also mapped the human *tar* gene (s) on the genome.

14. SUBJECT TERMS
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INTRODUCTION

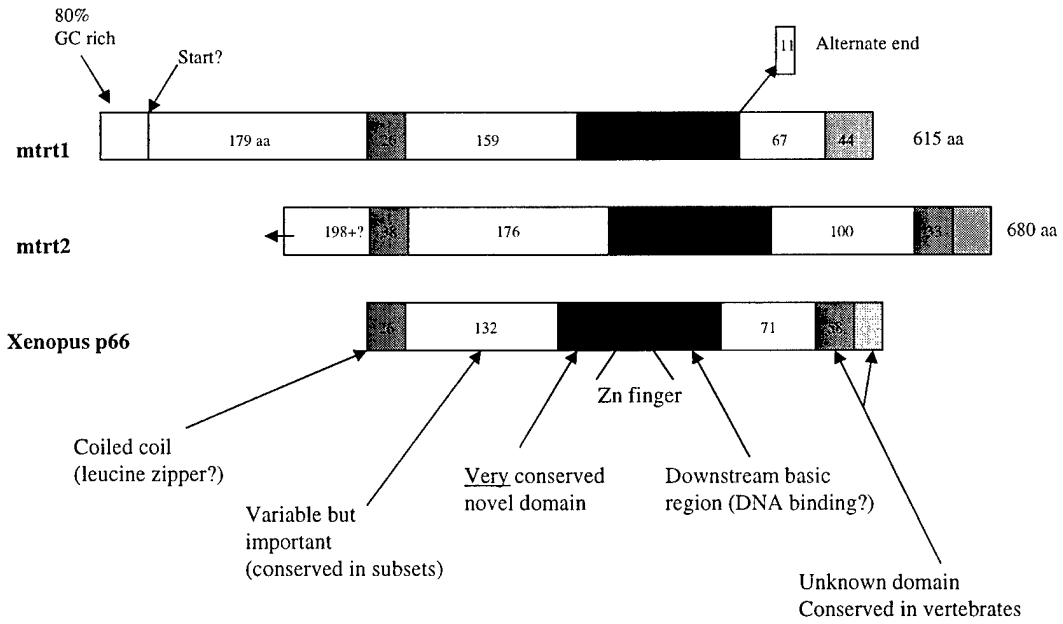
The goal of this grant has been to clone, map, and characterize the mammalian *tartaruga (tar)* gene in order to understand more about its function in cancer and, secondarily, in development. Our approach in the mouse (project 1) has been to study expression at the RNA level, and to map the genomic structure 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. In the previous year we cloned both mouse homologs of *tartaruga* and established the genomic organization of these genes. This year we built replacement vectors for both genes and we have generated chimeras for mouse *tartaruga 2.*

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 was completed and reported last year.

BODY

Project 1: The mouse *tar* genes

Knockout strategy:



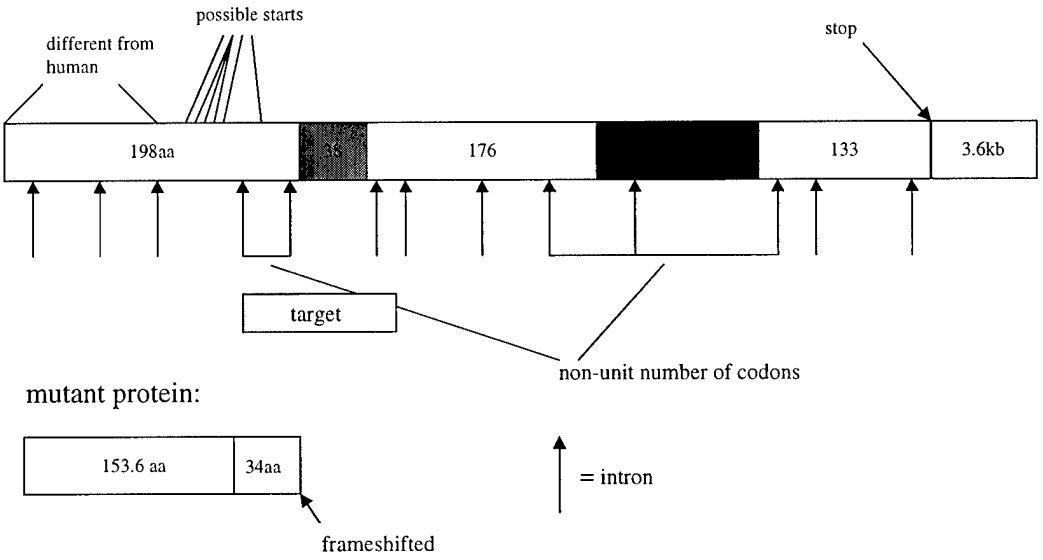
Tartaruga protein domains: mtrt1: mouse *tartaruga 1*; **mtrt2**: mouse *tartaruga 2* or mouse *p66;* **Xenopus p66** is the frog homolog of **mtrt2**, identified as a member of the NuRD or Mi-2 histone deacetylase complex (Wade *et al.*,Nature Genetics 23:62).

The *tartaruga* proteins contain two highly conserved regions, a 5' leucine zipper and a 3' zinc finger with conserved flanking regions.

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.

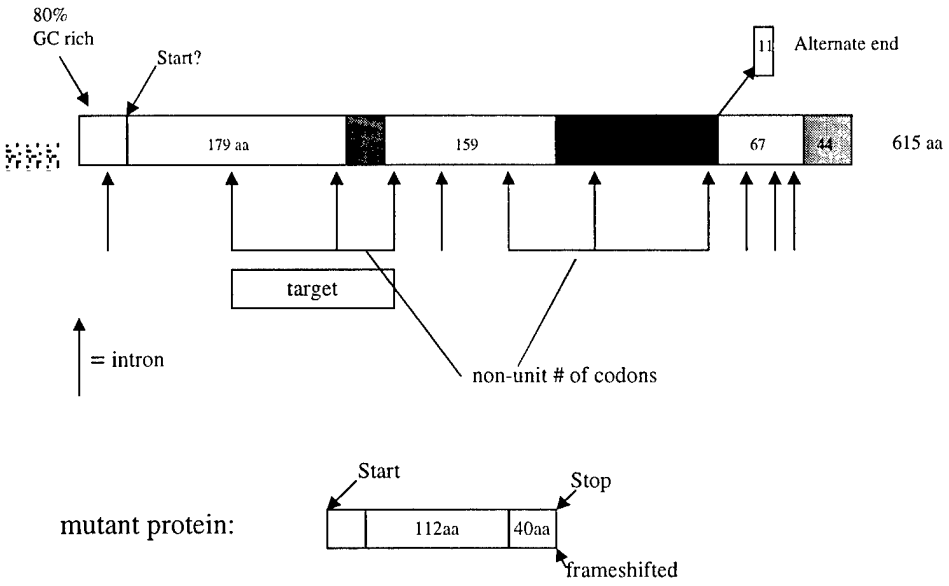
1.3 kb of DNA 5' of the three-exon target region and 6.3 kb of DNA 3' of the target region were removed from 129SV BACs to serve as homologous arms. These were inserted on either side of a floxed *pgkneo* cassette in the ploxPNT plasmid, obtained from Janet Rossant at University of Toronto. Distal to 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.

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 the animals will be crossed with C57BL mates in order to determine germline transmission of the ES cell line and mutation by coat color and tail biopsy, respectively.

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.

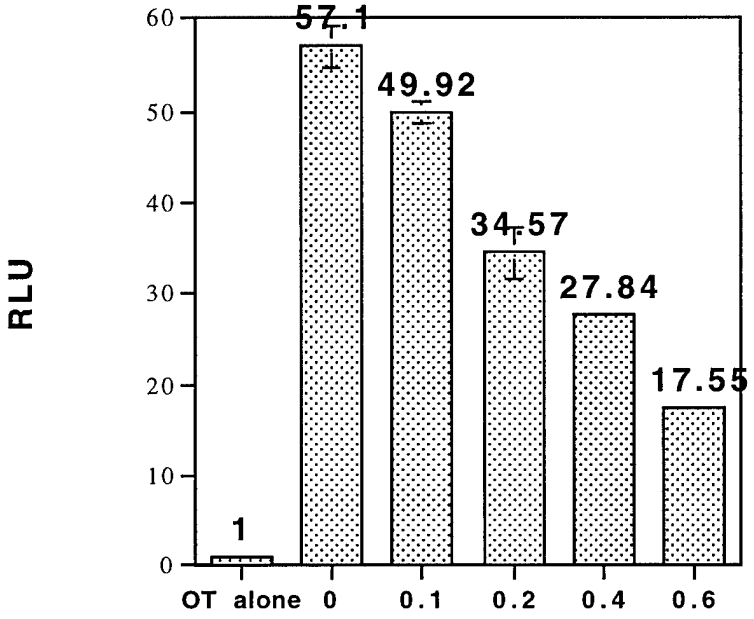
To assess recombination we developed both long range PCR and Southern blot assays as described above for *tartaruga 2*. Both long and short arms can be assessed on *BamH1* digests, due to the introduction of a *BamH1* site in the inserted region. Probes exterior to each homologous arm detect a 13.1 kb 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 200 clones so far that have survived selection. Of these, 150 appear to be negative by both PCR and Southern blot assays. We are presently analyzing the remaining 50.

trt inhibits β–catenin activation of a TCF reporter

Previous 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.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning mouse homologs of tartaruga
- Establishing genomic organization genes
- Building targeting constructs and generating chimeric mice with mutant cells.
- Establishing that tartaruga is a repressor of gene expression

REPORTABLE OUTCOMES

None

CONCLUSIONS

We are well on course to accomplish the 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. We do not expect major difficulties in achieving the ultimate goals.

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APPENDICES

None