Thus, the determinant of escape from rapid ER degradation may be proper folding of the hydrophobic TM peptide in the lumen of the ER, driven by the CYT region. The 40-aa-long CYT region of CD4, predicted to have a highly ordered structure, including an $NH_2$-terminal α-helical region (*17*), may induce proper folding of $\alpha_{tm}$. To explore this possibility, we replaced the CYT region of $CD4_{ex}$·$\alpha_{tm}$·$CD4_{cyt}$ with a 67-aa-long peptide that contains the intact $NH_2$-terminal 20 aa of the $CD4_{cyt}$, followed by 47 random aa created by a frame-shift mutation at $Gln^{421}$ of the CD4 cDNA ($CD4_{cyt.fs}$) (Fig. 1A). CD4 with this CYT region peptide was not susceptible to phosphorylation-dependent down-modulation, probably because of disrupted conformation in the first half of $CD4_{cyt}$ (*18*). $CD4_{ex}$·$\alpha_{tm}$·$CD4_{cyt.fs}$ was rapidly degraded without secretion (Fig. 4). Thus, whether the chimeric molecules were secreted or degraded was determined by the structure of the COOH-terminal cytoplasmic tail rather than by the length of the tail.

Thus, the $\alpha_{tm}$ contributes to translocation of TCRα into the ER lumen and to recognition by a lumenal degradative system. It is apparently incompetent to function as a TM region in the absence of association with other polypeptides. With a short CYT region, the $\alpha_{tm}$ may not fold properly in the lumen of the ER. Under these circumstances, TCRα would expose its hydrophobic TM region to the lumenal environment, resulting in rapid degradation. A putative soluble T cell suppressor factor is reported to contain TCRα in association with a second subunit (*19*). The structure of $\alpha_{tm}$ could have an important physiological consequence through the secretion of such a heterodimer.

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# Germ Line Transmission of a Yeast Artificial Chromosome Spanning the Murine $\alpha_1$(I) Collagen Locus


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**Molecular complementation of mutant phenotypes by transgenic technology is a potentially important tool for gene identification. A technology was developed that allows the transfer of a physically intact yeast artificial chromosome (YAC) into the germ line of the mouse. A purified 150-kilobase YAC encompassing the murine gene *Col1a1* was efficiently introduced into embryonic stem (ES) cells via lipofection. Chimeric founder mice were derived from two transfected ES cell clones. These chimeras transmitted the full length transgene through the germ line, generating two transgenic mouse strains. Transgene expression was visualized as nascent transcripts in interphase nuclei and quantitated by ribonuclease protection analysis. Both assays indicated that the transgene was expressed at levels comparable to the endogenous collagen gene.**


The size of many mammalian genes such as *NF-1*, *MDX*, or the gene encoding factor VIII is in the megabase range (*1–4*); this size range exceeds the limits of conventional cloning techniques, thus impeding the functional analysis of such genes in transgenic model systems. In addition to facilitating the study of known genes, the ability to genetically manipulate large pieces of DNA in mice would be useful for the identification of genes that have been localized by genetic and physical means to certain chromosomal regions. For example, if it were possible to transfer YACs (*5*) to the germ line of mutant mice, molecular complementation of the mutant phenotype would functionally identify genes or gene complexes present on the YAC, allowing the positional cloning and validation of new genes. Toward this goal, we have established procedures that permit the efficient transfer of physically intact YAC DNA into ES cells and we report the transmission of a gene locus carried on a 150-kb DNA fragment through the germ line.

As a model for establishing an in vivo complementation system, we have used the Mov13 mouse strain, which carries a molecularly and biologically well defined mutation. This strain was generated by random insertional mutagenesis after retroviral infection of early mouse embryos. A provirus had integrated into the gene for $\alpha_1$ (I) collagen (*Col1a1*), interfering with gene expression and resulting in an embryonic recessive lethal phenotype at day 13 of gestation (*6*).


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Attempts to molecularly complement the mutant phenotype by introducing the wild-type gene into the Mov13 germ line were not successful (*7*). The present study, in which the *Col1a1* gene was used as an experimental system, was initiated in an attempt to establish efficient procedures of introducing YAC DNA into the germ line.

The YAC clone Y22 was isolated from a *Mus spretus* library (*8*). The sequence divergence between M. *spretus* and M. *musculus* allows the discrimination between the experimentally introduced M. *spretus* DNA and the host M. *musculus* DNA by evaluating RFLPs in Southern blot hybridizations. The clone, spanning 150 kb surrounding the *Col1a1* locus, contained all known cis-acting regulatory sequences and conferred collagen expression to Mov13 mutant fibroblasts after stable transfection (*9*). The neomycin resistance gene under the control of the PGK promoter was an integral part of the left arm of the cloning vector pYAC151 (*8*) permitting drug selection of transfected cells. The Lt and Rt probes were derived from pBR322 sequences present in the left and right arm, respectively, by making a Bam HI–Pvu II digest of pBR322 and isolating the two fragments produced by this digestion.

In previous experiments we used DNA-lipid micelles to introduce the Y22 YAC DNA into fibroblasts (*9*). Transfection of ES cells with the same protocol proved to be highly inefficient and irreproducible. In most experiments where $10^9$ cells were used per transfection, no G418$^r$ colonies were obtained and only one transfection yielded seven drug-resistant colonies of which one clone (50.1) contained an intact YAC. To improve the efficiency of ES cell transfection, several modifications were incorporated into the original protocol (*10*). The DNA was isolated in 100 μM spermine and poly-L-lysine (3 to 5 μg/ml) was added prior to the addition of the cationic lipid *N*[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium methyl sulfate (DOTAP). The cells were exposed to the DNA-lipid complex in suspension instead of growing as monolayer. The modified protocol consistently yielded approximately 30 drug-resistant clones from $1 \times 10^8$ transfected cells representing approximately a 30-fold increase in transfection efficiency over the original method.

A total of 35 drug-resistant clones were isolated and analyzed. Southern blots were hybridized to probes from each vector arm (probes Lt and Rt) or to a collagen I cDNA probe (*9*) that spans the entire locus. Figure 1A shows the results obtained with two representative clones, 82.2 and 82.4. Hybridization of DNA digested with Bam HI, Pst I, or Pvu II to the collagen cDNA revealed several *Col1a1*-specific fragments that are polymorphic between M. *musculus* and M. *spretus* control DNA (lanes 3 and 4, respectively, middle panel). The M. *spretus*–specific fragments were detected in both transfected ES cell clones, indicating that the cells carried the donor *Col1a1* sequences. When the same blots were hybridized to the vector probes Lt and Rt, bands corresponding to the terminal fragments were detected. Bam HI has no recognition site in the vector arms and yielded a single fragment of different molecular size with either the Rt or the Lt probe. This suggests that the transgene had integrated at a single chromosomal site that was different in the two cell lines. Digestion with Pst I, which cleaves multiple times in the left arm and once in the right arm, generated the predicted internal fragments as well as a fragment specific for the flanking sequences of each integrated YAC DNA. Pvu II, which cleaves once in the left arm and has no recognition site in the right arm, generated fragments of a size consistent with a single YAC integration site in recipient DNA. Out of 35 drug-resistant clones, seven clones carried all three markers, five clones contained the collagen RFLP and the left arm but had no sequences

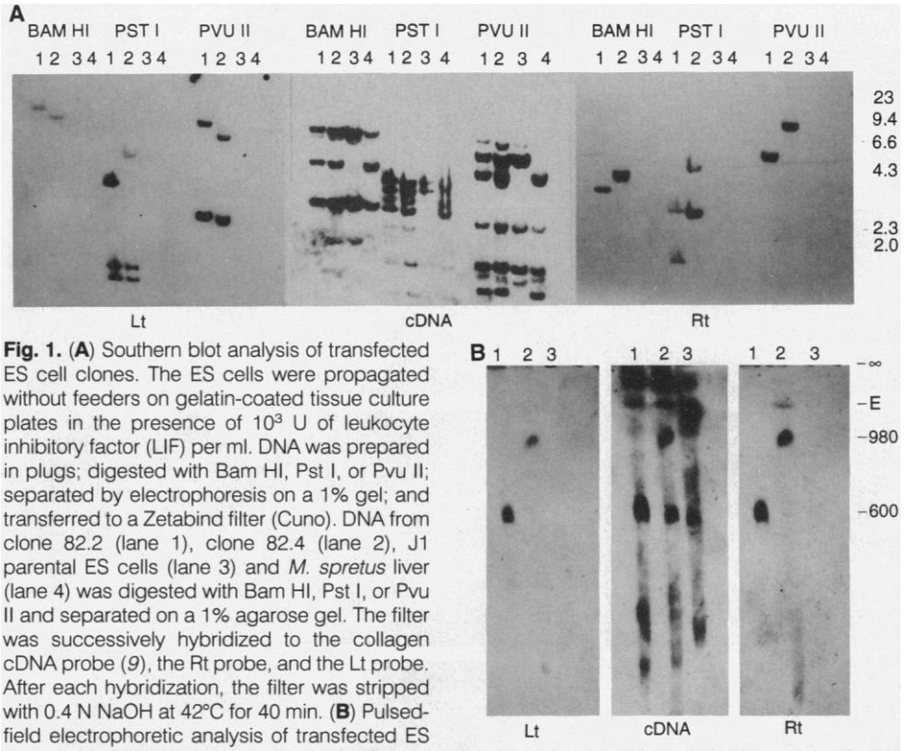


**Fig. 1.** (**A**) Southern blot analysis of transfected ES cell clones. The ES cells were propagated without feeders on gelatin-coated tissue culture plates in the presence of $10^3$ U of leukocyte inhibitory factor (LIF) per ml. DNA was prepared in plugs; digested with Bam HI, Pst I, or Pvu II; separated by electrophoresis on a 1% gel; and transferred to a Zetabind filter (Cuno). DNA from clone 82.2 (lane 1), clone 82.4 (lane 2), J1 parental ES cells (lane 3) and *M. spretus* liver (lane 4) was digested with Bam HI, Pst I, or Pvu II and separated on a 1% agarose gel. The filter was successively hybridized to the collagen cDNA probe (*9*), the Rt probe, and the Lt probe. After each hybridization, the filter was stripped with 0.4 N NaOH at 42°C for 40 min. (**B**) Pulsed-field electrophoretic analysis of transfected ES cell clones. DNA plugs were digested with Not I and separated on PFGE with a Bio-Rad CHEF DrII apparatus and ramped switching times from 30 to 140 s for 36 hours at 14°C. A Southern blot was prepared as above except that the gel was irradiated on a standard transluminator with 260 nm UV for 10 min prior to transfer to Zetabind (Cuno). The filter containing DNA from 82.2 cells (lanes 1), 82.4 cells (lanes 2), and J1 parental cells (lanes 3) was hybridized successively with the Lt, Rt, and collagen cDNA probes; ∞ marks the position of sample loading and E the exclusion zone of poor resolution.

**Table 1.** Generation of chimeric founder animals and germ line transmission of *Col1a1* YAC clone. Clones 50.1, 82.2, and 82.4 were microinjected into FVB, C57BL/6, or BALB/c blastocysts. Chimeric founder animals were bred with the respective host strain and germ line contribution of the ES cells was monitored by the presence of the dominant agouti coat color marker in the $F_1$ offspring. Tail DNA was isolated at weaning and tested for the presence of the transgene by PCR with *neo* primers (*12*) and by Southern analysis as described (Fig. 1A).

| Donor ES cell clone | Recipient blastocyst | Chimera frequency | Number of $F_1$ offspring | | |
|---|---|---|---|---|---|
| | | | Total born | Agouti coat color | YAC |
| 50.1 | FVB | 7/14 | 376 | 0 | 0 |
| | C57BL/6 | 7/18 | 220 | 0 | 0 |
| 82.2 | BALB/c | 8/18 | 375 | 130 | 31 |
| | C57BL/6 | 1/16 | 40 | 16 | 4 |
| 82.4 | BALB/c | 5/14 | 207 | 0 | 0 |
| | C57BL/6 | 3/16 | 222 | 9 | 7 |

hybridizing with the Rt probe, while the remaining 23 clones had only the Lt marker. This is consistent with our previous results (9) and indicates that the majority of drug-resistant clones contain partially deleted YAC clones.

Pulsed-field gel electrophoresis (PFGE) was performed to study whether the three markers Lt, collagen, and Rt detected by standard Southern blot analysis would co-localize on the same high molecular weight restriction fragment in Not I–digested DNA (Fig. 1B). Rt and Lt probes detected the same 600-kb fragment in clone 82.2. Similar analysis of clone 82.4 revealed a 980-kb fragment. Both the 600- and the 980-kb fragments were absent in the parental cell line. The collagen probe hybridized to the same fragment in the respective clone in addition to a 600-kb fragment, which was also present in the parental DNA. The strong hybridization signal at 600 kb in 82.2 DNA is consistent with two Not I fragments corresponding to the endogenous *Col1a1* Not I fragment as well as a transgene band. The 980-kb band corresponds to the transgene Not I fragment in 82.4 cells. These results suggest that the transgenes present in clones 82.2 and 82.4 were physically intact and were integrated at different chromosomal locations.

We used fluorescence in situ hybridization (FISH) to cellular DNA to confirm that the transgene copies were carried at a single chromosomal location. Metaphase spreads were simultaneously hybridized to a 24-kb genomic probe corresponding to *Col1a1* (detected by rhodamine staining) and to an 11-kb probe corresponding to the vector pYAC151 (8) (detected by fluorescein-avidin, Fig. 2). In both parental and transfected cell lines the collagen probe generated a hybridization signal at a single site at each of the two homologs of chromosome 11 corresponding to endogenous *Col1a1* (*11*). A third site of collagen-specific hybridization signal, which was located close to the centromere of a large chromosome in 82.2 cells and close to the telomere of a smaller chromosome in 82.4 cells, was seen in each of the transfected cell lines but not in the parental cells (Fig. 2). The latter chromosomal sites were also labeled by the vector probe in the respective transfected, but not in the parental, cells. These results indicate that vector and donor *Col1a1* sequences co-localize to a unique chromosomal site in the two transfected cell clones and, together with the Southern blot analyses described in Fig. 1, are consistent with each transgene being physically intact.

To test whether the transfected ES cell clones retained their developmental potential, clones 50.1, 82.2, and 82.4, each carrying a full length transgene, were injected into FVB, BALB/c, or C57BL/6 host blastocysts generating chimeric mice from all three clones. When crossed to animals of the re-

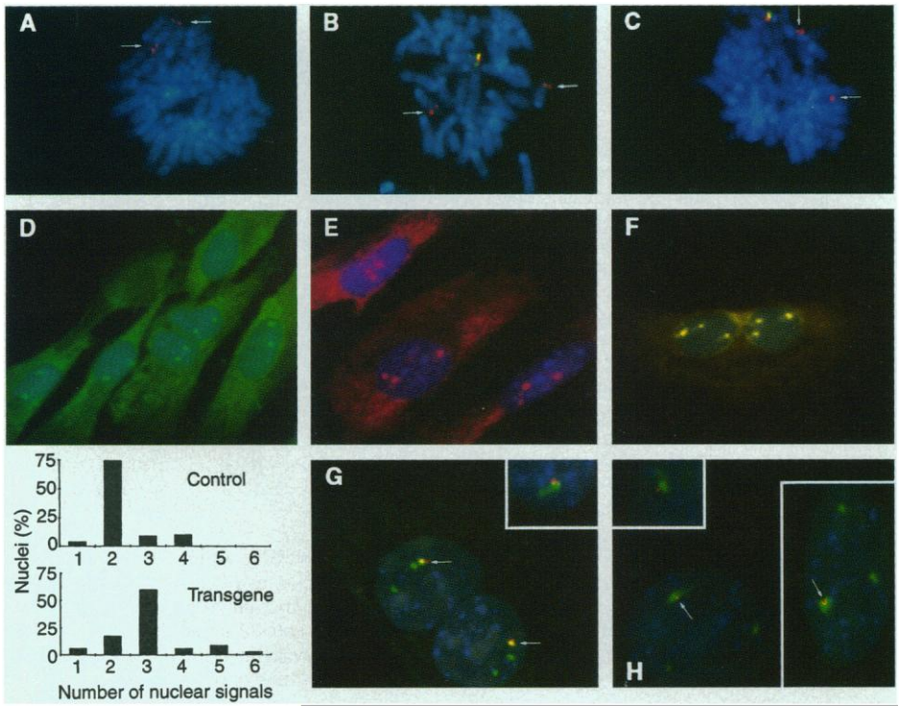


**Fig. 2.** Fluorescence hybridization analysis of transgene DNA and RNA. Cells were coded prior to hybridization and scored blindly. Total DNA was counterstained DAPI (blue). (**A** to **C**) DNA hybridization to metaphase preparations of embryonic stem cells. Digoxygenin-labeled *Col1a1* probe (red, arrows); biotin-labeled vector sequence probe (green-yellow signal). Cellular DNA was denatured prior to hybridization overnight in 50% formamide, 2× SSC at 37°C as described (*15, 31*). (**A**) Parental ES cell line J1 showing hybridization only to the two homologous collagen genes, with no detection of vector sequences. (B and C) 82.4 and 82.2 cells, respectively, each showing a third site of hybridization containing both vector and collagen sequences on different chromosomes. (**D** to **F**) Single probe hybridization to collagen RNA within paraformaldehyde-fixed monolayers of primary dermal fibroblasts, showing foci of concentrated nuclear RNA in addition to diffuse cytoplasmic RNA, detected with either fluorescein (D and F) or rhodamine (E). Under the conditions utilized, cellular DNA is unavailable for hybridization and RNA detection is promoted (*14, 15*). (D) Cells from nontransgenic control mouse showing two foci of collagen nuclear RNA. (E and F) Cells from the transgenic animal lines showing three foci of nuclear collagen RNA. Histogram shows percent of nuclei with different numbers of nuclear RNA foci in control and transgene populations. (**G** and **H**) Sequential hybridization first to collagen RNA (green) and then to vector (red), as described (*15*), in fibroblasts derived from transgenic animal. Cells of both transgenic lines show that one of the three sites of RNA signal is closely associated with vector DNA (arrow), marking the site of the transgene. Insets show higher magnification views of collagen RNA track with associated vector sequence.

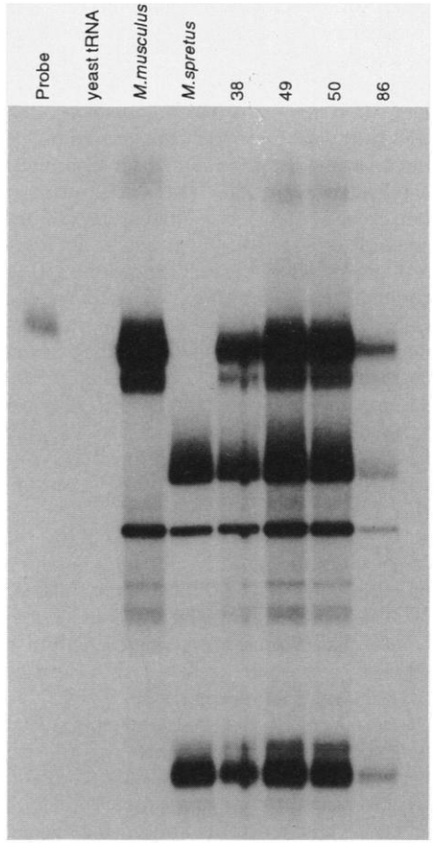


**Fig. 3.** RNase protection assay of transgene collagen mRNA (*32–34*). Total RNA was isolated from dermal fibroblasts. Lanes 3 and 4, control *M. musculus* and *M. spretus* cells. Lanes 5 to 8 contain RNA derived from transgenic lines 82.2 (#38, #49, and #50) and 82.4 (#86).

spective host background, chimeric founder animals derived from clones 82.2 and 82.4 but not from clone 50.1 transmitted the ES cell genome to their offspring as shown by the dominant coat color marker (Table 1). As determined by PCR analysis (*12*), 42 of 155 of these $F_1$ animals carried the transgene. Southern blot analysis similar to that described in Fig. 1A confirmed that the transgene carried the same molecular markers as in the donor ES cell clones 82.2 and 82.4, respectively (*13*).

Two different approaches were used to detect transgene-specific transcription in primary dermal fibroblasts prepared from transgenic mice. Nascent transcripts were visualized by FISH as localized foci or tracks (*14*), which have been shown to represent the site of transcription (*15*). As expected, the majority of the control cells (78%) revealed two large tracks of nuclear RNA indicating transcription on the two collagen alleles (Fig. 2D). In contrast, the majority of the transgenic cells (range 61 to 66%) showed three collagen tracks indicating collagen transcription at three nuclear sites (Fig. 2E, F, and histogram). To directly confirm that one of these foci was due to transgene transcription, sequential hybridization first to RNA and then to DNA (Fig. 2, G and H) showed that transgenic cells but not control cells yielded a vector-specific signal that co-localized with one of the three collagen transcription foci.

Results from direct visualization of nascent transcripts suggested that the transgene was expressed at a level similar to that of the endogenous gene. This was confirmed by quantitative RNase protection assay. A probe derived from the 3′ UT region of the collagen gene was hybridized to total cellular RNA isolated from dermal fibroblast cultures. This analysis detected a 317-bp fragment in *M. musculus* RNA and two fragments of 210 bp and 95 bp in *M. spretus* RNA (Fig. 3). All three fragments were seen in RNA isolated from four fibroblast cultures derived independently from the transgenic mice. The intensity of the transgene-specific fragments was similar to that of the host bands confirming that the transgene was expressed at a similar level as the endogenous gene.

Other methods of introducing YAC-sized DNA into mammalian cells have been used, including spheroplast fusion and microinjection (*16–21*), but no successful transfection of ES cells has been reported to date. Spheroplast fusion introduces the whole yeast genome into the transfected cells. Because DNA injected into mammalian embryos is highly mutagenic, it is possible that the presence of yeast DNA in the transfected ES cell interferes with their ability to contribute to the germ line. The use of gel-purified DNA in ES cell transfections is consequently advantageous. The transfection of DNA-lipid micelles resulted in a large fraction of the drug-resistant clones carrying an intact transgene and did not interfere with totipotency of the manipulated ES cells.

Our results demonstrate that large fragments of DNA, after transfection of ES cells with DNA-lipid micelles, can be efficiently introduced into the mouse germ line. The collagen gene carried on the YAC was expressed at normal levels at two different chromosomal sites. Transfer of YAC-sized DNA into the germ line should be a valuable tool for manipulating large genes in the animal and helping define their cis-regulatory elements. Also, it would be of considerable interest to explore the frequency of homologous recombination of YAC-sized DNA segments that are isogenic with the recipient ES cell (*22*). The most important use of the procedure may be molecular complementation, which should, in combination with the extensive physical and genetic maps (*23–29*) already at hand, prove to be a powerful approach to gene identification. The procedure should be of considerable utility for identification of genes that are altered in mutant mouse strains and that have been mapped genetically, but for which physical markers are rare. Molecular complementation of the mutant phenotype by candidate YAC clones that have been localized to the mutant locus would constitute a functional assay for identification of the gene.

*Note added in proof*: Insertion of YACs has been achieved by two other groups (*30*).

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33. Total RNA (approximately 10 μg/lane) was hybridized with the uniformly labeled probe p141 (317 bp Eco RI–Hind III fragment spanning the last exon and portion of 3′UT for murine α1(I) collagen [K. Mooslehner, K. Harbers, *Nucl. Acids Res.* **16**, 773 (1988)], $2 \times 10^5$ count/min per assay) in a volume of 20 μl containing 80% deionized formamide, 40 mM Hepes, pH 7.9, and 400 mM NaCl. The samples were denatured at 68°C for 5 min and incubated at 50°C for 18 hours. Unhybridized probe was digested at room temperature for 1 hour with 50 μg/ml RNase A and 2 μg/ml RNase $T_1$ in a volume of 300 μl buffer (containing 10 mM Tris, pH 7.5, 5 mM EDTA, 300 mM NaCl). The samples were then treated with proteinase K (0.3 mg/ml), 0.6% SDS at 37°C for 30 min, phenol extracted, ethanol precipitated, and separated by electrophoresis in a denaturing 6% acrylamide gel containing 7 M urea.
34. Preparation of primary dermal fibroblasts from either control (*M. musculus* or *M. spretus*) or experimental mice (38, 49, 50, 86). Dorsal skin was shaved, removed, finely minced, suspended in 1% crude collagenase (CLS-2, Worthington), and digested for 120 min at 37°C The digested tissue was pipetted up and down 10 times, pelleted, and resuspended in 5 ml of 0.25% trypsin-EDTA and digested for 60 min at 37°C. Tissue was pipetted up and down 20 times and then pelleted. The pellet was resuspended in 50 ml of 15% fetal bovine serum, 1% penicillin-streptomycin-fungizone in DMEM, and plated in 5× 10-cm tissue culture dishes. After cells grew to confluence, cultures were split and logarithmically growing cells were used for FISH or harvested for RNA by guanidine isothiocyanate lysis.


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