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THE ROLE OF ACTIVIN SIGNALING IN TESTICULAR GROWTH AND DIFFERENTIATION

SUBMITTED BY:

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I. Original Specific Aims

A. INTRODUCTION AND HYPOTHESIS

The majority of activin receptor IIA (ActRIIA)-deficient mice are viable and demonstrate reproductive defects secondary to suppressed follicle stimulating hormone (FSH) levels. The mutant male mice display a delay in fertility, have decreased testis size and reduced sperm number but are fertile. Similarly, FSH-deficient male mice have decreased testis size, reduced sperm number and motility and are fertile. Because ActRII is expressed both in pituitary gonadotropes (where it regulates FSH homeostasis) and in the testis, it has not been possible to delineate the direct role of ActRIIA signaling within the testis, independent of influencing the FSH levels. **Based on these observations, we hypothesize that characterization of male reproductive phenotypes in FSH and ActRIIA double mutant mice will give important insights into the role of activin signaling via ActRIIA in testicular growth and differentiation.** This hypothesis will be addressed in the present proposal.

B. SPECIFIC AIMS

The specific aims of this proposal are as follows:

Specific Aim 1: Generate double homozygous mutant mice that are deficient in both FSH and ActRIIA by a genetic intercross.

We have earlier generated independent strains of mice lacking either ActRIIA or FSH. A minority of ActRIIA-deficient mice die at birth due to craniofacial defects. However, the majority of these mice are viable and live to adulthood and display reproductive phenotypes. We have also engineered a mutation at the unique FSHβ locus (FSH is an α:β heterodimer, the β-subunit is hormone-specific) in embryonic stem cells and generated FSH-deficient mice. FSH-deficient mice are viable; mutant male but not female mice are fertile. We will intercross FSH-deficient male mice (fertile) and heterozygous ActRIIA female mutant mice (homozygous female mice are infertile) to obtain double heterozygous and eventually double homozygous mutant male mice.

Specific Aim 2: Characterize the role of ActRIIA signaling in testicular growth and function.

Based on our analyses with *in vivo* mouse models and those of others with *in vitro* experiments, activin signaling in the testis is important for normal male reproductive function. We will determine the direct consequences of absence of ActRIIA signaling (and in the absence of FSH) in testicular function by analyzing the FSH/ActRII double mutant male mice and comparing them to the individual mutant mice that lack either ActRIIA or FSH alone. In particular, we will examine the fertility, analyze morphologically and histologically, the testes from these mice, we will evaluate the consequences of absence of FSH and activin receptor IIA signaling on sperm number and motility, we will quantitate testosterone levels in serum and testes, and finally we will determine if Sertoli cell proliferation is affected in the absence of both FSH and ActRIIA in these mice.

II. Summary of Data Obtained

Specific Aim 1: Generate double homozygous mutant mice that are deficient in both FSH and ActRIIA by a genetic intercross

Following a two generation breeding scheme, we generated a number of double heterozygous male and female mice. These mice were initially set up for mating to obtain double homozygous mutant mice. Subsequently, we have also set up matings between FSH-/-, ActRIIA+/- or FSH+/-, ActRIIA-/- males and double heterozygous female mice to increase the frequency of generating the double homozygous mutant mice. These data are summarized below in Table 1.

Table 1. Genetic crosses.

Genetic cross #	Male		Female		# of cages set up	# of litters generated	# of double mutant males obtained
	FSH	ActRIIA	FSH	ActRIIA			
1	+/-	+/-	+/-	+/-	7	42	6
2	-/-	+/-	+/-	+/-	19	135	22
3	+/-	-/-	+/-	+/-	6	30	3

During the analysis of these mating schemes, for nearly 1 year now, we have observed that Genetic cross #3 did not yield efficient litter sizes, hence the majority of the double mutant males were obtained through Genetic Crosses #1 and #2. All mice were genotyped by Southern blot analysis using external probes (Fig. 1).

Specific Aim 2: Characterize the role of ActRIIA signaling in testicular growth and function

1) Fertility analysis. The body weights of double mutant males are comparable to that of double heterozygous or wild-type male mice. We have set up 6 independent mating pairs at 42 days to test the fertility of these mice. Interestingly, 6/6 of these double mutants were fertile despite the absence of two major signaling pathways in the testis, *i.e.*, FSH and ActRIIA. However, as shown below in Table 2, the litter size is significantly reduced when compared to that obtained from double heterozygous crosses.

Table 2. Breeding data.

Male		Female		Litter size	
FSH	ActRIIA	FSH	ActRIIA	Mean ± SEM	# of litters
+/-	+/-	+/-	+/-	5.0 ± 0.2^{a}	42
-/-	-/-	+/-	+/-	3.0 ± 0.1^{b}	26

a vs. b, $p<0.05$ (t-test)

2) Morphological and histological analyses. As expected, the testis size is decreased in double mutant males, compared to wild-type controls. However, contrary to what we hypothesized, the testis size did not decrease significantly further in the double mutants when compared to ActRIIA mutation alone, at 42 days. The testis weight data of various genotype combinations are shown in Table 3.

Table 3. Testis weights at 42 days

FSH	ActRIIA	Testis weight (mg) Mean ± SEM	# of mice
+/+	+/+	88.3 ± 2.5	12
+/-	+/+	80.1 ± 1.7[c]	32
-/-	+/+	32.8 ± 0.8	37
+/+	+/-	71.9 ± 3.6[d]	6
+/+	-/-	22.6 ± 1.0[a]	6
+/-	+/-	59.3 ± 2.5[e]	12
-/-	+/-	33.5 ± 1.9	10
+/-	-/-	27.8 ± 1.4	12
-/-	-/-	23.7 ± 1.1[b]	8

a vs. b, $p>0.05$ (t-test); c vs. e or d vs. e, $p < 0.05$ (t-test)

We have fixed the testis samples from several double mutants in Bouin's reagent overnight and processed for histology. A cross-section view is representatively shown in Figure 2. In general, the histological analysis indicates that all of the spermatogenesis stages appear normal, although the tubular volume is decreased in the testis of double mutants. In addition, normal Leydig cells are evident in these sections. Furthermore, we have independently processed testis samples from 5 double mutant male mice at 42 days. These samples were sent to Dr. Nigel Wreford, Monash University, Australia. They are currently being analyzed using stereochemical and digital imaging techniques for additional quantitative data on various testicular cell types.

3) Sperm parameter evaluation. To analyze the functional consequences of the combined absence of FSH and ActRIIA signaling in the testis, we have quantitated the epididymal sperm number from male mice at 42 days, by the established methods of our laboratory. Although histological analysis indicated that the double mutant testis contained sperm in the lumen, as can be seen in Table 4, the double mutant mice contained significantly lower number of sperm compared to either FSH- or ActRIIA-deficient mice in the epididymis. Surprisingly, the mutant male mice were fertile despite this massive reduction in sperm number. This further suggests, lack of both FSH and ActRIIA might be affecting the germ cell numbers either directly or indirectly.

Table 4. Epididymal sperm number (x 10^6)

FSH	ActRIIA	Mean ± SEM	n
-/-	+/+	1.3 ± 0.04a	6
+/+	-/-	1.5 ± 0.02b	5
-/-	-/-	0.4 ± 0.01c	5

a vs. c, $p<0.05$; b vs. c, <0.05 (t-test)

4) Expression analyses of ActRIIB. In order to evaluate the expression of ActRIIB, the other type II activin receptor in the absence of ActRIIA, in the testis of double mutant male mice, we performed Northern blot analysis on the total RNA isolated from wild-type, ActRIIA-deficient and double homozygous mice deficient in both FSH and ActRIIA. The blos were probed with a mouse ActRIIB cDNA probe, stripped and reprobed with a 18S RNA control probe. Figure 3 clearly represents that ActRIIB message was not upregulated in the absence of ActRIIA signaling. Therefore, we can conclude that activin RIIB signaling does not compensate for the absence of activin RIIA signaling pathway in the mouse testis. In addition, we are also currently

performing *in situ* hybridization experiments to confirm the expression of ActRIIB in the mouse testis.

5) Serum and intratesticular testosterone analysis. We have obtained serum samples and prepared testicular extracts from 42 day old, wild-type, double heterozygous, FSH-deficient, ActRIIA-deficient, and double homozygous mutant mice. The testicular extracts were prepared by following the protocol standardized in the laboratory of Dr. Marvin L. Meistrich, M.D. Anderson Cancer Center. All of these samples were kept frozen at -20°C. These will be tested in the solid phase testosterone RIA that has been well standardized in my laboratory. The assay can be completed within 3 hours. We are collecting a sufficient number of samples to set up a large scale assay. We anticipate to finish this experiment before June 30, 2000. Based on the normal Leydig cells in the testis and normal accessory glands in the double mutant mice, we predict that the serum levels of testosterone may not be altered. However, it will be interesting to analyze the intratesticular testosterone levels which may be reduced in the double mutant mice. Furthermore, we have analyzed serum FSH levels using a rat FSH RIA and found that they were not significantly different between FSH +/-, ActRII +/- and FAR +/-, +/- male mice. As expected, no values were obtained with FSH -/- or FAR -/-, -/- male mice, in the absence of circulating FSH in these mice.

6) Sertoli cell proliferation assay. In order to assess the labeling index of Sertoli cell proliferation kinetics, we are currently generating more double mutant mice and mice deficient in only ActRIIA or FSHβ. We intend to obtain testis samples from bromodeoxyuridine-injected mutant mice between 7-10 days, the period during which Sertoli cells are known to rapidly proliferate. We expect, based on our germ cell data, that no differences will be obvious between double mutant or single mutant mice.

Together, the abovementioned morphological, histological, molecular and functional analyses (status summarized in Table 5) suggest that, locally, activin signaling via ActRIIA pathway within the testis is important for maintenance of normal germ cell numbers and for fertility. The generation and characterization of FAR double mutant mice clearly are useful to further delineate this signaling pathway.

The future experiments will aim at 1) examining the expression of important cell-specific markers in the testis of double mutant mice; 2) to genetically rescue the ActRIIA-deficient mice by directed expression of Smad-2 to Sertoli cells; and 3) to further identify the downstream targets of this signaling pathway within the mouse testis.

Table 5. Summary of status of experiments.

Experiment	Status	Anticipated date of completion
Fertility analysis	Completed	—
Morphological and histological analysis	Completed	—
Sterelogical analysis	Final stages	June 30, 2000
Sperm parameter evaluation	Completed	—
Expression analysis of activin RIIB	Northern blots: completed In situ: ongoing	June 15, 2000
Testosterone RIA	More samples to be collected	June 20, 2000
FSH RIA	Completed	—
Sertoli cell proliferation assay	Currently generating 7-10 day- old mutant mice	June 30, 2000

III. Figure Legends

Figure 1: Genotyping of mice by Southern blot analysis. Tail DNA was isolated by proteinase-K digestion overnight at 55°C. ~5 μg of DNA was digested with appropriate restriction enzymes, separated on 0.7% agarose gels, transferred to GeneScreen plus membranes and hybridized at 37°C overnight with ^{32}P-labeled random primed probes. The blots were washed and exposed to autoradiography film at -80°C for 36 hrs. and developed. The enzymes used were: SacI/BamHI for FSH; EcoRI for ActRIIA. For FSH mutation: WT allele = 7.5 kb; mutant allele = 3.6 kb. For ActRIIA mutation: WT allele: 8.0 kb; mutant allele = 11.4 kb. A representative blot is shown. All the expected size bands are clearly seen for each mutation.

Figure 2. Histolgical analysis of a FAR double mutant male mouse testis. Testis was surgically removed from a double mutant male mouse at 42 days, fixed overnight in Bouin's reagent, washed extensively in Li_2CO_3-saturated 70% ethanol at RT, paraffin embedded 5 μm sections were cut and stained with hematoxylin-PAS reagent. Multiple tubules are seen in this section, abundant late stage spermatids are seen in the lumen (asterisk). All stages of spermatogenesis appear grossly normal. Arrows denote Leydig cells.

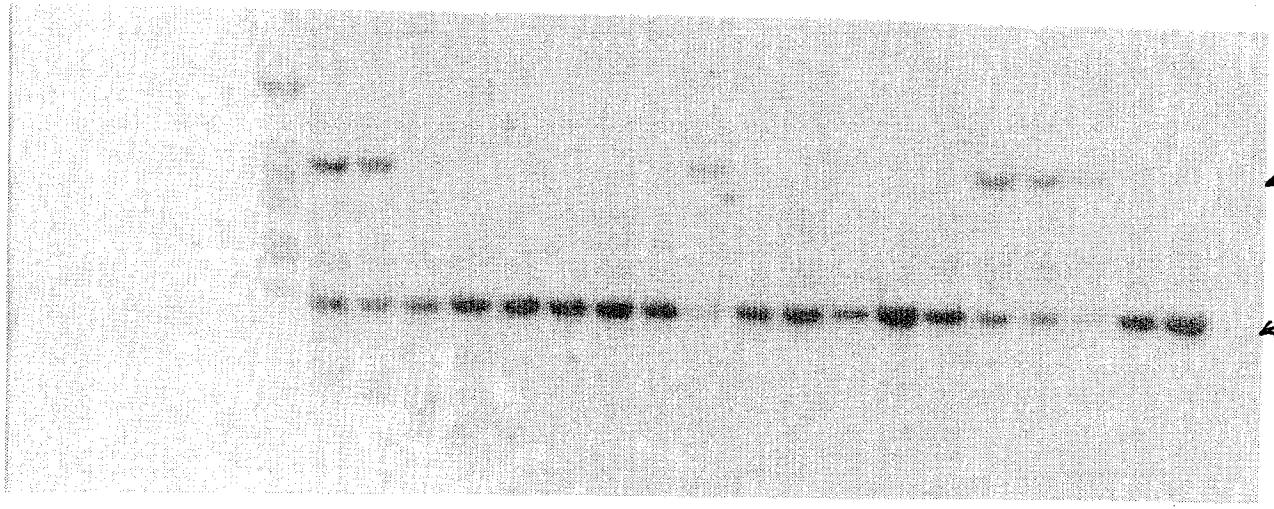
Figure 3. Northern blot analysis of testis RNA. Total RNA from 42 day old wild-type, ActRIIA-deficient and FSH/ActRIIA double mutant mice was prepared by TRIZOL method, denatured and separated on 1.4% formaldehyde-agarose gels, transferred to nylon membrane and hybridized with an ActRIIB cDNA probe. The blot was later stripped and rehybridized with an 18S RNA probe. As can be seen, in the absence of ActRIIA signaling, the ActRIIB mRNA was not upregulated in FAR -/- mice. Further, preliminary *in situ* hybridization analysis confirms the similar expression in FAR -/- testis.

IV. Research Collaborations Initiated

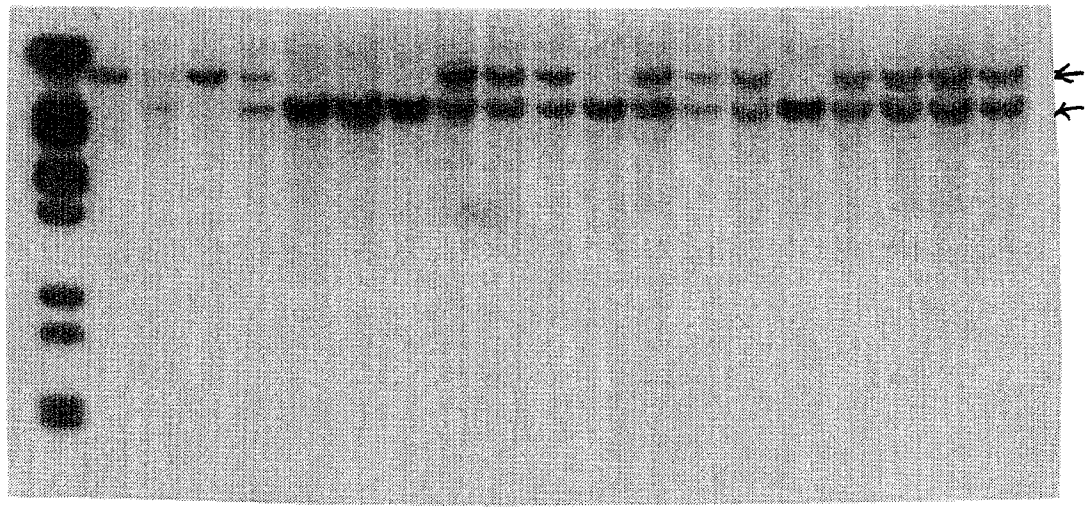
During the course of this project, we have initiated important research collaborations with the following laboratories:

1) Dr. Marvin L. Meistrich, M.D. Anderson Cancer Center, Houston, TX (Testicular testosterone assays)
2) Dr. Teresa K. Woodruff, Northwestern University, Evanston, IL (Directed expression of SMAD-2 to Sertoli cells)
3) Dr. Nigel Wreford, Monash University, Australia (Stereological analysis of testicular cell types)

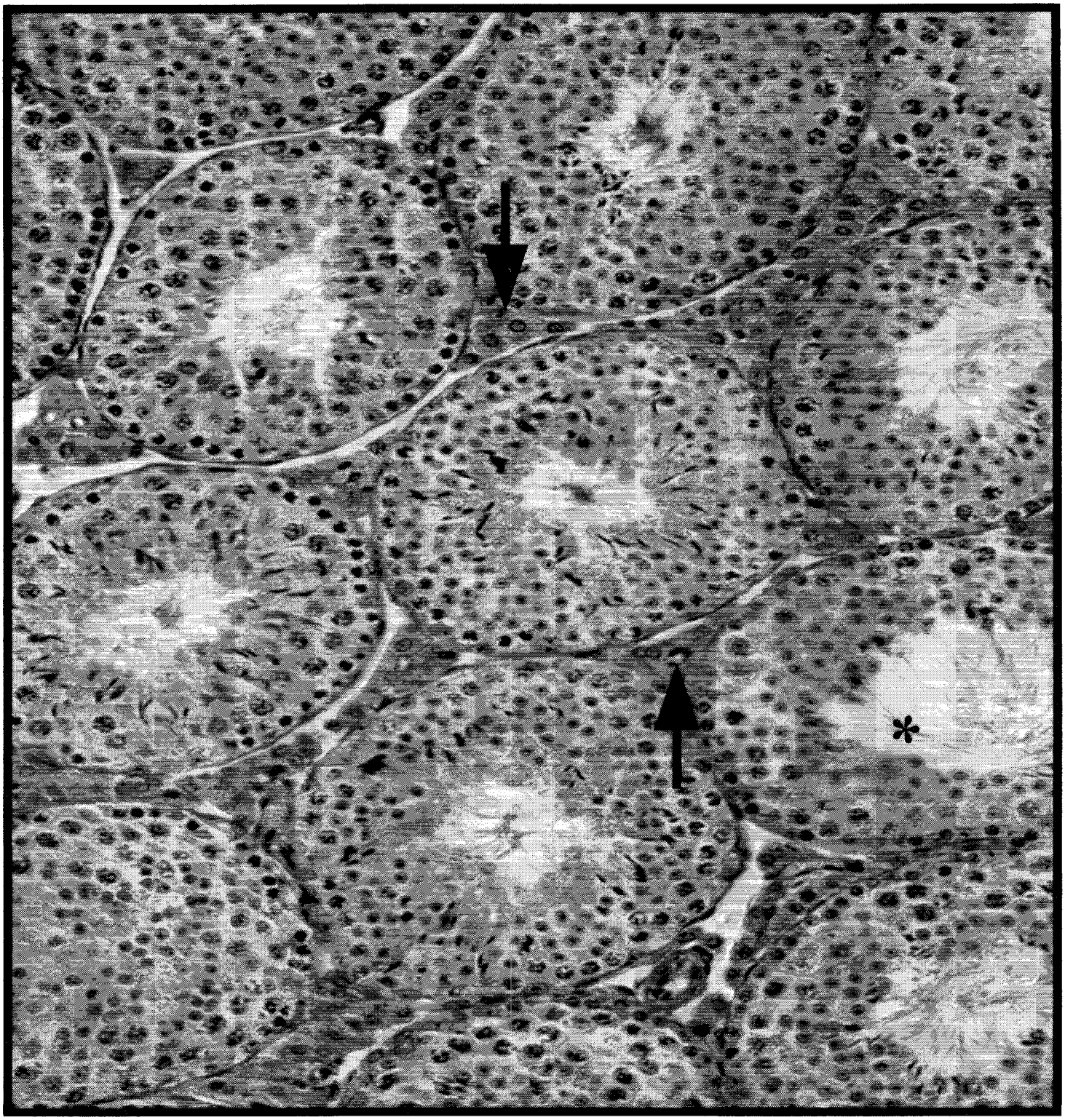
FSH



ActRIIA



Kumar, T.R.
Figure - 1



Kumar, TR
figure - 2

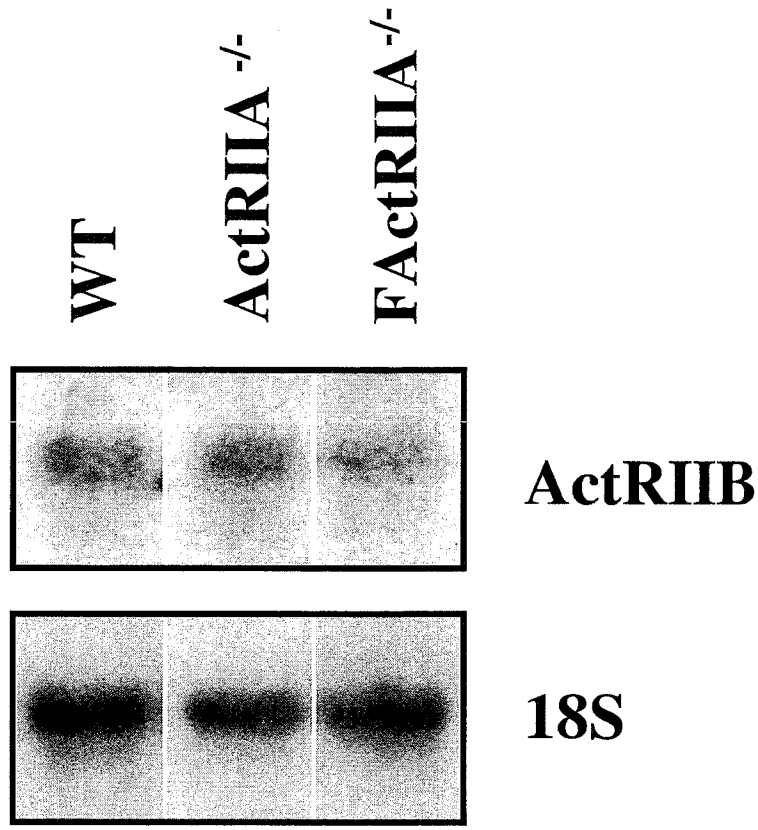


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Figure 3