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# Androgen-Induced *Rhox* Homeobox Genes Modulate the Expression of AR-Regulated Genes

**Abbreviated title: *Rhox5*-Regulated Genes**

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**ABSTRACT**

*Rhox5,* the founding member of the reproductive homeobox on the X chromosome (*Rhox*) gene cluster, encodes a homeodomain-containing transcription factor that is selectively expressed in Sertoli cells, where it promotes the survival of male germ cells. To identify *Rhox5*-regulated genes, we generated 15P-1 Sertoli cell clones expressing physiological levels of *Rhox5* from a stably transfected expression vector. Microarray analysis identified many genes altered in expression in response to *Rhox5*, including those encoding proteins controlling cell cycle regulation, apoptosis, metabolism, and cell-cell interactions. Fifteen of these *Rhox5*-regulated genes were chosen for further analysis. Analysis of *Rhox5*-null male mice indicated that at least 9 of these are *Rhox5*-regulated in the testes *in vivo*. Many of them have distinct postnatal expression patterns and are regulated by *Rhox5* at different postnatal time points. Most of them are expressed in Sertoli cells, indicating that they are candidates to be directly regulated by *Rhox5*. Transfection analysis with expression vectors encoding different mouse and human *Rhox* family members revealed that the regulatory response of a subset of these *Rhox5*-regulated genes is both conserved and redundant. Given that *Rhox5* depends on AR for expression in Sertoli cells, we examined whether some *Rhox5*-regulated genes are also regulated by androgen receptor (AR). We provide several lines of evidence that this is the case, leading us to propose that RHOX5 serves as a key intermediate transcription factor that directs some of the actions of AR in the testes.

**Introduction**

Homeobox genes encode transcription factors harboring a 60 amino-acid DNA-binding motif called a homeodomain (HD). These transcription factors govern key embryonic developmental processes, including body-axis formation, organogenesis, and limb development (1). While the roles of homeobox genes in embryonic development have been intensely studied for over two decades, their functions in controlling post-embryonic developmental events have only begun to be examined. Homeobox genes are good candidates to control post-embryonic events, as many homeobox genes are expressed during postnatal development and in adult tissues. Indeed, mouse knockout studies have shown that the *HoxA9, Hoxc13,* and *Pdx1* homeobox genes are crucial for hematopoiesis, hair growth, and gut homeostasis, respectively (2-4).

Another post-embryonic process likely to be controlled by homeobox genes is spermatogenesis. This idea is supported by the fact that over 50 of the ~200 known mouse homeobox genes are expressed in the testis and other male reproductive organs (5-12). While this suggests a role for homeobox transcription factors in male reproduction, the functions of most homeobox transcription factors in the postnatal and adult male reproductive tract are not yet clear, as efforts to elucidate their roles using knockout mice have been clouded by putative functional redundancies and embryonic lethality (6). These efforts have identified some homeobox genes that control the formation of the male reproductive tract during embryogenesis, but the identity of homeobox genes that have roles in the male reproductive tract after birth have remained largely obscure (5, 12-14).

To our knowledge, the only mammalian homebox gene demonstrated to have a role in spermatogenesis is *Rhox5* (previously known as *Pem*), the founding member of the reproductive homoeobox genes on the X chromosome (*Rhox*) gene family (7). All the members of the *Rhox* gene family form a tandem array on the X chromosome in mammals. The mouse *Rhox* gene cluster contains 33 members, and thus is the largest homeobox gene cluster known in any species (7-11, 15). Virtually all of the *Rhox* genes are selectively expressed in reproductive tissues and the placenta, suggesting that they encode transcription factors devoted to regulating gametogenesis and embryogenesis (7).

*Rhox5* was first identified in a search for genes differentially expressed between two related T-lymphoma cell clones that exhibited marked differences in developmental status and tumorigenicity (16). *Rhox5* was initially of interest because it was found to be selectively expressed in T-cell lymphomas, not normal T cells, and also in a wide variety of other tumor cells of diverse developmental and tissue origins (17). Later investigations revealed that *Rhox5* encodes a tumor antigen (18), interacts with protein involved in tumorigenesis (19, 20), and its expression is induced by the proto-oncogene Ras (21, 22). While these studies have suggested the possibility that *Rhox5* has a role in tumorigenesis, its precise role in malignancy has not yet been determined. Other studies have focused on *Rhox5's* normal expression pattern and biological roles. *Rhox5* is normally expressed in the early mouse embryo in a developmentally regulated manner (23), and is later expressed in placenta (24, 25) and in both male and female germ cells in developing fetal gonads (26, 27). In neonatal and adult mice and rats, *Rhox5* expression is primarily confined to somatic cells in the testis, epididymis, and ovary (28, 29). In the testis, its expression is restricted to Sertoli cells, where it is expressed

in a stage-specific manner during the seminiferous epithelial cycle by an androgen- and androgen receptor (AR)-dependent mechanism (30, 31).

The expression of *Rhox5* in somatic cells in the male reproductive tract is crucial for normal spermatogenesis and sperm maturation. Targeted mutation of *Rhox5* in male mice leads to subfertility, marked by increased germ-cell apoptosis, reduced sperm number, and reduced sperm motility (7). *Rhox5*-null mice exhibit an increased frequency of apoptotic germ cells in seminiferous epithelial cycle stages that normally contain dying germ cells (stages I to IV and stage XII) and they also have apoptotic germ cells in stages that normally lack dying germ cells (stages V to XI). *Rhox5* is unlikely to act directly on germ cells to control germ cell survival and motility, as it is not detectably expressed in germ cells but instead is expressed in Sertoli cells, the nurse cells in intimate contact with germ cells (29, 30). This suggests a model in which *Rhox5* functions in spermatogenesis by virtue of its ability to regulate genes encoding Sertoli cell secreted and cell surface proteins that, in turn, control key events in germ cells, including their differentiation and survival (32-34).

To begin to address the molecular mechanisms by which *Rhox5* helps drive spermatogenesis, we used transcriptional profiling to identify genes regulated by physiological levels of *Rhox5* in 15P-1 cells, a well-established Sertoli cell line (35-37). This genome-wide analysis led to the discovery of many *Rhox5*-regulated genes, a large proportion of which encode cell-surface proteins. Many of these were expressed in Sertoli cells, which was of interest because such genes could mediate *Rhox5*-induced signals that impinge on germ cells. Also identified were *Rhox5*-regulated genes encoding other kinds of proteins, including secreted factors, transcription factors, and metabolic enzymes. We

focused on a subset of these genes and found that most are also *Rhox5*-regulated in the testes *in vivo*. We determined the testicular cell types and developmental stages in which the regulation of each gene occurs. We also identified other *Rhox* family members that regulate their expression, and provided evidence that the *Rhox*-dependent regulatory circuitry controlling these genes is conserved. Finally, we addressed whether any of the *Rhox5* gene targets are secondary androgen-response (SAR) genes. These are long sought-after genes that are not directly regulated by androgen and AR, but rather are controlled by androgen-dependent regulators, such as androgen-induced transcription factors (38, 39). Because *Rhox5* is an AR- and androgen-inducible gene in Sertoli cells, it is a good candidate to regulate SAR genes. Indeed, we obtained many lines of evidence that several *Rhox5*-regulated genes are SAR genes. We also showed that other androgen-inducible *Rhox* genes (besides *Rhox5*) are capable of regulating these candidate SAR genes. The discovery of genes coregulated by AR and RHOX transcription factors has the potential to begin illuminate the molecular mechanisms by which androgens drive spermatogenesis.

**Results**

**Establishment of Sertoli cell clones expressing physiological levels of *Rhox5***

To identify *Rhox5* target genes by a gain-of-function approach, we screened Sertoli cell lines for those that express low endogenous *Rhox5* mRNA levels. Our rationale was that a low *Rhox5*-expressing line would provide an optimal baseline for identifying genes regulated by physiological levels of *Rhox5* expressed from a *Rhox5*-expression vector. We predicted that most Sertoli cell lines would express low levels of *Rhox5*, as we previously found that

primary mouse Sertoli cells dramatically downregulate *Rhox5* mRNA expression after only 1

day in culture (30). Indeed, we found that all three Sertoli cell lines that we tested—15P-1,

MSC-1, and Tm4—expressed very low levels of *Rhox5* mRNA, as judged by quantitative (Q)

polymerase chain reaction (PCR) analysis (Supplemental Fig. S1). The two cell lines with

the lowest expression, 15P-1 and Tm4, expressed ~300-fold less *Rhox5* mRNA than adult

testes. This is a conservative estimate of the difference given that *Rhox5* mRNA is only

expressed in Sertoli cells in the adult testis (26, 29). Indeed, RHOX5 protein was

undetectable in 15P-1 cells, as judged by Western blot analysis (Supplemental Fig. S2). We

chose 15P-1 over Tm4 for our gain-of-function study because we found that 15P-1 cells could

be transfected more efficiently than Tm4 cells (using Lipofectamin; data not shown). We

also chose the 15P-1 cell line because it exhibits many normal Sertoli cell characteristics,

including the expression of genes normally expressed by Sertoli cells (e.g., WT1 and Steel)

and the ability to support the transit of male germ cells through meiosis (35, 40, 41).

To express *Rhox5* in 15P-1 cells, a murine *Rhox5* full-length cDNA was cloned into an

expression vector under the control of the CMV immediate early promoter. This construct

was then stably transfected into 15P-1 Sertoli cells and several independent G418-resistant

subclones were obtained. Northern blot analysis demonstrated that many of the cell clones

expressed *Rhox5* mRNA at levels comparable to that in postnatal and adult testes (data not

shown). To confirm that *Rhox5* was expressed, we employed Western blot analysis. This

showed that the stably transfected cell clones also expressed RHOX5 protein (Fig. 1A). To

obtain a quantitative assessment of *Rhox5* mRNA levels, qPCR analysis was employed (Fig.

1B). This demonstrated that clones 6 and 24 expressed the highest level of *Rhox5* mRNA

and thus these 2 clones were chosen as the "Rhox5-positive" clones for microarray analysis.

Clones 14 and 16, which had the lowest level of *Rhox5* mRNA (comparable to the level in

parental 15P-1 cells; data not shown), were chosen as the "Rhox5-negative" clones for

microarray analysis.


**Identification of genes regulated by *Rhox5* in Sertoli cells**

Total cellular RNA was isolated from clones 6, 24, 14 and 16 and analyzed by microarray

analysis.  The digitally captured data was analyzed using GeneSpring software to determine

the genes that were differentially expressed between the four samples in all pairwise

combinations.  This analysis revealed that 182 genes were either upregulated or

downregulated by 2-fold or more in the *Rhox5*-positive cell clones as compared to the *Rhox5*-

negative cell clones (Supplemental Tables 1 and 2).  The *Rhox5*-regulated genes that we

identified by this approach encode proteins encompassing a broad range of functions.  We

used OntoExpress software to classify them and put them into categories based on gene

ontology classification (42).  Supplemental Figure 3 provides a graphical representation of

the molecular function analysis, biological process analysis, and cellular component analysis

for the proteins encoded by all 182 genes.   Supplemental Figs. 4 and 5 provide the same type

of information for only the Rhox5-upregulated and -downregulated genes, respectively.

Compared to the whole mouse genome, the *Rhox5*-regulated genes were not statistically

overrepresented in any one category but rather were involved in a wide array of molecular

and cellular functions.

***In vitro*** **regulation and cellular site of expression** *in vivo*

To verify the regulation observed by microarray analysis, we performed qPCR analysis on 15 of the 182 genes (Table 1). Nine of them encode membrane-bound proteins, which we chose because such proteins could mediate *Rhox5*-dependent signaling between Sertoli cells and germ cells. The other 6 genes were selected for various reasons. Carboxypeptidase X1 (*Cpxm1*) and ganglioside-induced differentiation associated protein 1 (*Gdap1*) were chosen because they are dramatically regulated by *Rhox5*, according to our microarray analysis (Table 1). BTB and CNC Homology 2 (*Bach2*) and kruppel-like factor 9 (*Klf9*) encode transcription factors with well-established functions; the latter is known to have a role in female reproduction and neuronal development (43, 44). Lastly, isocitrate dehydrogenase 3 (*Idh3a*) and 2,3-bisphosphoglycerate mutase (*Bpgm*) were selected because they encode proteins directly involved in metabolism. This was of interest because we have found that *Rhox5* regulates many other genes involved in metabolism (determined using an independent approach to the one we took here; MacLean J, Hu Z, and Wilkinson MF, unpublished observations).

To verify the regulation of these 15 genes, qPCR was performed on total cellular RNA from the four original 15P-1 clones used for microarray hybridization (6, 24, 14, and 16), as well as other 15P-1 stable clones expressing different levels of *Rhox5* mRNA (clones 1, 4, 11, 19, and 26; data not shown). This analysis demonstrated that all 15 of the transcripts that we tested were regulated in precisely the same manner as indicated by microarray analysis (Table 1). Furthermore, the magnitude of the regulation for most of the transcripts was similar when measured by either qPCR analysis or microarray analysis.

To assess which of these 15 genes are candidates to be direct targets of *Rhox5*, we assessed whether they are expressed in Sertoli cells, the site of *Rhox5* expression (29). To do this, we generated an enriched Sertoli cell fraction from adult testes using a modified protocol involving gravity sedimentation and enzymatic treatments (see Materials and Methods). This procedure also generates purified interstitial (mainly Leydig) cells, which allowed us to determine whether any of these *Rhox5*-regulated genes are also expressed in this somatic cell subset. Hypotonic shock was used to lyse most of the contaminating germ cells in both Sertoli- and interstitial-enriched fractions (45). By comparing gene expression in Sertoli- and interstitial cell fractions prepared with or without hypotonic shock, we were able to assess the contribution of germ cells. Although it is not possible to generate entirely pure populations of these cell types, our protocol yielded fractions that were substantially enriched, based on their relative expression of Sertoli (*Gata1* and *Rhox5*) (29-31, 46) and Leydig cell markers (luteinizing hormone (LH) receptor [*Lhr*]) (47). To assess the effectiveness of hypotonic shock in removing germ cells, we assessed the levels of two germ cell-specific genes: testicular orphan receptor-2 (*Tr2*) and Deleted in Azoospermia-like (Dazl) (48, 49).

Table 2 shows the expression of the 15 *Rhox5*-regulated genes in the different cell subsets, as assessed by qPCR analysis. Five of the genes were predominantly expressed in Sertoli cells (group 1), based on two criteria. First, they were more highly expressed in the Sertoli cell fraction that underwent hypotonic shock and than the one that did not (under the headings "Sertoli cells" and "Sertoli no-shock," respectively). Second, they were more highly expressed in the purified Sertoli cell fraction than in total testes (the latter of which

was given a value of "1"). Of note, however, we found that the magnitude of the increased expression in the purified Sertoli cell fraction (relative to total testes) varied considerably between genes. This may, in part, be the result of some of the genes being expressed in other cell types in the testes. Another possible contributing factor is that our procedure may not equally purify Sertoli cells in all stages of the seminiferous epithelial cycle. This would lead to different levels of enrichment, depending on the pattern of expression of a given gene during the seminiferous epithelial cycle. For example, the positive control, *Gata1*, has been shown to be expressed exclusively by Sertoli cells but because it exhibits stage-specific expression (50), this could explain why we found its expression was increased by less in the purified Sertoli cell fraction (~4-fold) than one of the *Rhox5*-regulated genes, interferon receptor-2 (*Ifnar2*), which exhibited ~8-fold increased expression. As another example, the *Rhox5*-regulated genes, *Tfc*, which encodes the transferrin receptor, was previously shown to be specifically expressed only by Sertoli cells in the testes (51), but we found it was only expressed at ~2-fold higher levels in the purified Sertoli cell fraction than in total testes. We suggest that this may be explained by its stage-specific expression (52). We did find that *Tfc* expression was relatively Sertoli cell-specific, as *Tfc* mRNA was at ~10-fold higher levels in the purified Sertoli cell fraction than in the purified Leydig cell fraction.

We divided the other 9 *Rhox5*-regulated genes into 3 categories: (i) those expressed in both the Sertoli- and interstitial-cell fractions (group 2), (ii) those expressed predominantly in the interstitial-cell fraction (group 3), and (iii) those predominantly expressed in testicular cell types other than Sertoli cells or interstitial cells (group 4). The group-2 genes are candidates to be directly regulated by *Rhox5*, while those in groups 3 and 4 probably are indirectly

regulated through paracrine signaling from Sertoli cells. We note, however, that *Bpgm*, which is in group 4 because it was less expressed in the Sertoli and interstitial fractions than in total testes, exhibited significantly increased expression in purified Sertoli cells than in non-shocked Sertoli cells, which suggests that it is expressed in at least a subset of Sertoli cells. Together, these results provide evidence that ~60% of the *Rhox5*-regulated genes that we selected for in-depth analysis are expressed in Sertoli cells in the adult testes *in vivo*.

### *In vivo* regulation

While the 15P-1 cell line has many Sertoli cell characteristics (35, 37), it is an immortalized cell line adapted to tissue culture and thus it does not completely mimic normal Sertoli cells. Hence, it was crucial to determine whether the genes regulated by *Rhox5* in 15P-1 cells were also regulated by *Rhox5 in vivo*. We took 3 approaches to address this question. First, we compared their expression in adult testes from *Rhox5*-null and control mice. Second, we compared their expression in purified somatic cell fractions from *Rhox5*-null and control adult testes. Third, we compared their postnatal pattern of expression in *Rhox5*-null and control mice. The first approach revealed that 5 of the 15 genes were significantly regulated by *Rhox5* in the adult testis: cluster-w4 antigen (*Cd24a*), transmembrane protein (*Tmem*) *176a*, *Tmem176b*, *Klf9*, and *Cpxm1* (Fig. 2). All 5 of these genes were upregulated in the *Rhox5*-null adult testes, indicating that they are negatively regulated by *Rhox5 in vivo*. Consistent with this, 4 of these genes were repressed by *Rhox5* in 15P-1 cells (Table 1). The lone exception, *Cd24a*, was strongly upregulated by *Rhox5* in 15P-1 cells, whereas it was modestly

downregulated by *Rhox5* in the adult testes. Three of the genes—*Tmem176a, Tmem176b,* and *Klf9*—were downregulated by similar magnitudes in 15P-1 cells and the adult testis.

Because molecular analysis of the whole testes will often not reveal genes regulated by *Rhox5* in only a specific subset of testicular cells, we also examined *Rhox5*-mediated regulation in adult testicular cell subsets. Because we found that most of the *Rhox5*-regulated genes are expressed in the enriched Sertoli cell and interstitial cell fractions, we examined these two cell subsets. Figure 3 shows the ratio of gene expression in these two fractions prepared from Rhox5-*null* and control mice (note that the ratio is the average of two independent experiments and is presented on a log scale). The data indicate that several genes are regulated by *Rhox5* in the Sertoli cell fraction, the interstitial cell fraction, or both. Of the 5 genes up- or down-regulated by 2-fold or more in Sertoli cells (indicated with an "a" in Fig. 3, upper panel), 4 were regulated in the same manner in response to *Rhox5* in 15P-1 cells (Table 1). The lone exception, Frizzled homolog 2 (*Fzd2*), was upregulated by *Rhox5* in the Sertoli cell fraction *in vivo* and modestly downregulated by *Rhox5* in 15P-1 cells. However, *Fzd2* expression was downregulated by *Rhox5* in the interstitial fraction, indicating that it is regulated in the same manner by *Rhox5* in interstitial cells *in vivo* as it is the 15P-1 cell line. Three of the other 4 genes regulated by 2-fold or more in the interstitial cell fraction (indicated with an "a" in Fig. 3, lower panel) were also regulated in the same manner *in vitro* (Table 1). Together, this data indicated that 4 additional genes (beyond those identified in total adult testes) are *Rhox5* regulated *in vivo*: *Fzd2*, *Gdap1*, *Pltp*, and *Tmem47* (also known as *Tm4sf10*). All 4 of these genes are expressed in purified Sertoli cells (Table

2) and regulated by *Rhox5* in Sertoli cells (Fig. 3), indicating that they are all candidates to be direct targets of RHOX5.

As a final approach to assess the regulation of these 15 genes by *Rhox5 in vivo*, we examined their expression pattern during the first wave of spermatogenesis in *Rhox5*-null and control mice. This approach provided an independent assessment of *Rhox5*-mediated regulation and it had the potential to pinpoint the stage(s) of spermatogenesis at which *Rhox5* acts on its target genes. This analysis demonstrated that 5 of the 15 genes were significantly regulated by *Rhox5* at one or more time points during the first wave of spermatogenesis (Fig. 4 and Supplemental Fig. S6). While some of these genes were regulated by *Rhox5* only transiently during the first wave of spermatogenesis (i.e., at very specific postnatal time points), the regulation was reproducible. Furthermore, all 5 of these genes exhibited the same regulation *in vitro*. Thus, 3 of the genes (*Fzd2, Unc5c*, and *Tmem176a*) were downregulated by *Rhox5* both *in vivo* and in 15P-1 cells, whereas the other 2 genes (*Cd24a* and *Gdap1*) were upregulated by *Rhox5* both *in vivo* and in 15P-1 cells (Fig. 4 and Table 1). Three of the genes (*Fzd2, Gdap1*, and *Tmem176a*) were also regulated in adult testes in the same manner as they were in postnatal testes (Figs. 2 and 3). Four of the genes were expressed and/or regulated in purified Sertoli cells (Table 2 and Fig. 3), indicating that they are candidates to be direct targets of *Rhox5*.

Together, the data derived from these 3 different approaches indicated that 10 of the 15 genes regulated by *Rhox5* in the 15P-1 Sertoli cell line are also regulated by *Rhox5* in the testis *in vivo*. Seven of these 10 are expressed in the enriched Sertoli cell fraction and thus may be directly regulated by *Rhox5* (Table 2).

### *Rhox5* targets *Ar*-regulated genes

The *Rhox5* gene depends on the nuclear hormone receptor AR and androgen for expression in Sertoli cells (see Introduction and Discussion). This led us to hypothesize that a subset of *Rhox5*-regulated genes are also regulated by AR and androgen. In other words, we hypothesized that a subset of *Rhox5*-regulated genes are secondary androgen-responsive (SAR) genes that are androgen regulated through the action of *Rhox5*. As a first test of this hypothesis, we determined whether any of the 15 *Rhox5*-regulated genes described above are regulated by AR *in vivo*. To do this, we examined their expression in Sertoli cell AR-knockout (*Scarko*) mice, which have *Ar* mutated conditionally in Sertoli cells (53), and testicular feminized (*Tfm*) mice, which have a naturally occurring mutation in the *Ar* gene that inactivates AR function (54). qPCR analysis of adult testes from these mutant mice revealed that 11 of the 15 *Rhox5*-regulated genes had significantly altered expression compared to control testes (Fig. 5). Eight of these genes were regulated in the same manner by *Ar* and *Rhox5* (either positively or negatively in response to both), indicating that these are candidate SAR genes. Six of them had significantly altered expression in both *Scarko* and *Tfm* mice, providing strong evidence that these genes are *bona fide* AR-regulated genes. The other two genes, *Fzd2* and *Pltp*, were only regulated in *Scarko* mice, not *Tfm* mice. While there are many possible explanations for this, a likely one is that because *Tfm* mice are cryptorchid (55); the abnormally high temperature of the testes in these mice prevents normal AR-mediated regulation of these two genes.

The signature feature of SAR genes is that they do not directly respond to AR (39, 56).

To determine if the 8 candidate SAR genes have the potential to respond to AR, we examined

their 5′ and 3′ flanking regions for androgen-response elements (AREs) using the

Gemomatix program. Four of these genes had no consensus AREs, while the other 4 had

either 1 or 2 consensus AREs (Supplementary table 3). To empirically determine whether

the 8 candidate SAR genes do not directly respond to AR, we examined whether their

expression was altered in response to AR and androgen under conditions when *Rhox5* is *not*

induced. This was accomplished using 15P-1 cells, which does not exhibit increased *Rhox5*

mRNA levels in response to the testosterone analog R1881 and transfection with an AR

expression plasmid (Supplementary Fig. S6). We found that AR and R1881 did not

significantly affect the expression of any of the 8 candidate SAR genes in the 15P-1 cell line

(Supplemental Fig. S7). In contrast, AR and R1881 did increase the expression of the AR-

regulated gene *Fabp* (data not shown).

As another test of AR/androgen responsiveness, we generated a luciferase reporter

construct containing 3 kb of 5′ flanking sequence from the candidate SAR gene *Unc5c* (Fig.

6A). We chose *Unc5c* because it harbors no consensus AREs in this region (Table 3 and

data not shown). When contransfected into the MSC1 Sertoli cell line with an AR

expression plasmid and incubated with R1881, this *Unc5c* promoter construct did not exhibit

altered luciferase expression (Fig. 6B). In contrast, the positive control, a luciferase reporter

construct containing the *Rhox5* proximal promoter (*Pp*) (Fig. 6A), expressed greatly elevated

levels of luciferase in response to AR and R1881 (Fig. 6B). Note that while the *Rhox5 Pp*

reporter was greatly increased in expression in response to AR and R1881, the endogenous

*Rhox5* gene was not significantly elevated in expression (as judged by qPCR analysis; data not shown), probably because it is in a repressed state due to DNA methylation (17, 57). The ability of *Unc5c* to respond to AR only under conditions in which *Rhox5* expression is also induced (Figs. 5 and 6), coupled with the ability of *Unc5c* to be regulated by *Rhox5* both *in vitro* and *in vivo* (Table 1 and Fig. 4, respectively), provides strong evidence that *Unc5c* is regulated by AR through the action of *Rhox5*.

**Identification of common targets of AR-regulated *Rhox* genes**

In the previous section, we provided evidence that we had identified SAR genes that are regulated by AR through the action of the AR-inducible gene *Rhox5*.  As another means to address whether we identified *bona fide* SAR genes, we determined whether they are regulated by other AR-inducible *Rhox* genes.  We previously demonstrated that *Rhox2, Rhox3*, *Rhox10*, and *Rhox11* are all strongly upregulated in expression in response to AR and androgen in Sertoli cells (7).   Thus, we examined whether any of these other AR-inducible *Rhox* genes were capable of regulating the putative SAR genes we had identified.  The putative SAR genes that we selected for this analysis were those that fulfilled 3 criteria: (i) regulated by *Rhox5 in vivo*, as judged by our 3-prong analysis (Figs. 2-4), (ii) regulated by AR *in vivo* (Fig. 5), and (iii) significantly expressed in the purified Sertoli cell fraction (Table 2, category 1 or 2).   The 6 genes that fulfilled these criteria were *Gdap1, Tmem176a, Tmem176b, Fzd2, Pltp*, and *Unc5c*.   To determine whether these 6 genes are regulated by AR-regulated *Rhox* genes, we transiently transfected expression vectors encoding RHOX2, RHOX3, RHOX5, RHOX10, and RHOX11 into 15P-1 cells (we previously showed that all

these RHOX proteins are all expressed at similar levels from these expression vectors (32).

qPCR analysis revealed that each of the 6 SAR genes was significantly regulated in response to at least 2 of the AR-inducible *Rhox* genes (Fig. 7A). For example, *Unc5c* was negatively regulated by *Rhox3, Rhox5*, and *Rhox10*. *Tmem176a* and *Tmem176b* were also regulated by *Rhox3* and *Rhox5*, but instead of being regulated by *Rhox10*, these two genes responded to *Rhox11*. *Pltp* had the same pattern of expression as *Tmem176a* and *Tmem176b,* except that *Rhox2* also negatively regulated its expression. *Fzd2* had an unusual pattern of expression in that it was negatively regulated by *Rhox2, Rhox3, Rhox5*, and *Rhox11*, but was positively regulated by *Rhox10*. To assess specificity, we also evaluated the effect of *Rhox8*, which is not AR regulated, either as assessed in a Sertoli cell line (7) or in postnatal and adult testes *in vivo* (MacLean J., de Gendt K, Verhoeven G., and Wilkinson M.F., unpublished observations). We found that forced expression of *Rhox8* did not significantly affect the expression of any of the SAR genes except for *Gdap1*. *Rhox8* positively regulated *Gdap1*, as did *Rhox5* and *Rhox10*. Of note is that we found that transiently transfected *Rhox5* only modestly regulated the SAR genes (Fig. 7A), which contrasted with its more dramatic effects on some of the SAR genes in cells stably transfected with *Rhox5* (Table 1; i.e., *Gdap1, Pltp,* and *Unc5c*). Regardless of this quantitative difference, *Rhox5* had the same qualitative effect on the 6 SAR genes in transiently and stably transfected cells (i.e., *Rhox5* negatively regulated all the SAR genes except for *Gdap1* under both conditions).

To determine whether the regulation of the putative SAR genes is a conserved response, we examined the effect of forced expression of human *RHOXF1* and *RHOXF2*. These are the only human *RHOX* genes that have been characterized; both are selectively expressed in

human testes (58, 59). *RHOXF1* is an androgen-induced gene, while *RHOXF2* has not been characterized in this regard (58). We found that transient transfection of expression vectors encoding either *RHOXF1* or *RHOXF2* triggered the downregulation of *Unc5c* and *Pltp* (Fig. 7B). *RHOXF2*, but not *RHOXF1*, upregulated *Gdap1* expression, while *RHOXF1*, but not *RHOXF2*, modestly decreased the expression of both *Tmem176* and *Tmem176b*. The only putative SAR gene that did not significantly respond to either *RHOXF1* or *RHOXF2* was *Fzd2*. We conclude that human *RHOXF1* and *RHOXF2* share with androgen-regulated mouse *Rhox* family members the ability to regulate most of the SAR genes, suggesting that this is a conserved response.

**Discussion**

Homeobox genes were originally identified over 25 years ago and have since been shown to control a wide variety of developmental and cellular events from yeast to mammals, yet surprisingly little is known about the gene networks downstream of homeobox genes. The discovery of the *Rhox* homeobox gene cluster provides an opportunity to identify gene networks important for male and female gametogenesis. In this report, we focused on identifying genes downstream of *Rhox5*, the founding member of the *Rhox* gene cluster. This Sertoli cell-expressed gene promotes the survival of mouse male germ cells in all stages of the seminiferous epithelial cycle (7). *Rhox5* is also required to generate normal levels of motile spermatozoa and for normal male fertility (7). To identify physiologically relevant genes downstream of *Rhox5* that might mediate these functions, we took a two-prong approach in which we first identified *Rhox5*-regulated genes using genome-wide expression

profiling in a Sertoli cell line and then followed that up with *in vivo* analysis. We favored

using a Sertoli cell line for our initial screen of *Rhox5*-regulated genes for several reasons.

First, this allowed us to identify regulated genes in a relatively homogenous set of cells. By

contrast, analysis of whole testes and testicular cell subsets has potential problems with

cellular heterogeneity and cellular contamination, respectively. Second, the Sertoli cell line

we chose for our analysis, 15P-1, expresses little or no *Rhox5* mRNA or RHOX5 protein

(Supplementary figs. 1 and 2, respectively), which allowed us to take a simple gain-of-

function approach to identify *Rhox5*-regulated genes. Third, we carefully selected stably

transfected 15P-1 cell clones that expressed normal levels of *Rhox5* to allow us to screen for

physiologically relevant *Rhox5*-regulated genes. Finally, 15P-1 is an ideal cell line to

perform molecular studies on the molecular mechanisms by which RHOX5 regulates its

target genes, as these cells are transiently transfected efficiently and they exhibit many normal

Sertoli cell characteristics, including many Sertoli cell markers and the ability to support the

differentiation of germ cells (35-37, 40, 41).

Using this approach, we identified many genes regulated by *Rhox5* (Table 1),

including those encoding proteins involved in a wide variety of functions (Supplementary

table 1 and 2). We focused on 15 of these genes by first verifying that they are regulated by

*Rhox5* in 15P-1 cells (Table 1) and then conducting an in-depth analysis of their expression

pattern and regulation in response to *Rhox5 in vivo* (Figs. 2-4). By analyzing (i) adult testes,

(ii) purified cell fractions from adult testes, and (iii) postnatal testes from many time points

during the first wave of spermatogenesis from both *Rhox5*-null and control littermate mice,

we found that at least 11 of these 15 genes are regulated by *Rhox5 in vivo* (Table 1, Figs. 2-4).

Seven of these 11 *in vivo* Rhox5-regulated genes are expressed in Sertoli cells, based on our cell-subset fractionation analysis (Table 2), and thus are candidates to be directly regulated by *Rhox5*. Direct analysis of whether they are direct targets is complicated by the need for a ChIP-grade RHOX5 antisera and the fact that the *cis* elements responsible for regulation could be far from their promoter regions. We showed that many of these Sertoli cell-expressed genes are androgen- and/or AR-regulated (Fig. 5) and thus they may mediate some of the actions of androgen in the testes, as discussed in more detail below. Interestingly, 6 of these 7 genes encode cell-surface proteins and thus they may be involved in cell-cell signaling between Sertoli cells and/or between Sertoli and developing germ cells.

While some *Rhox5*-regulated genes appeared to be expressed and regulated exclusively in Sertoli cells, many *Rhox5*-regulated genes were expressed and/or regulated in other testicular cell types. For example, we found that a number of the *Rhox5*-regulated genes were expressed in both the Sertoli and interstitial cell fractions (Table 2, category 2); some of these were regulated by *Rhox5* in the interstitial cell fraction (Fig. 3). We also identified a gene exclusively expressed in the interstitial cell fraction—*Cd24a* (Table 2, category 3)—that exhibited modestly altered expression in *Rhox5*–null mice (Figs. 3 and 4), suggesting that *Cd24a* is regulated by *Rhox5* in a paracrine manner. Other *Rhox5*-regulated genes were expressed at very low levels in both the Sertoli and interstitial cell fractions (Table 2, category 4) and thus these genes may be regulated by *Rhox5* in other testicular cell types, such as germ cells or myoid cells. The overall picture that emerges from this analysis of 15 *Rhox5*-regulated genes is that even though RHOX5 protein is restricted to Sertoli cells (30, 31), its web of regulation extends to various cell types in the testes.

Because *Rhox5* is an androgen- and AR-induced homeobox gene (29-31), it is a candidate to encode a transcription factor that mediates some of the actions of androgen in the testes. This predicts that a subset of RHOX5 targets are SAR genes; i.e., genes indirectly regulated by AR through the action of intermediate factors (60). While several SAR genes have been defined in the prostate (39, 61), to our knowledge none have been defined in the testes. It is crucial to define SAR genes in the testis, as such genes are likely to be mediators of androgen action in this male reproductive organ. Progress on this front has been stymied by the fact that very few androgen-regulated transcription factors have been identified in the Sertoli cell, the only cell type definitively known to mediate androgen action in the testes (6). To our knowledge, the only androgen-induced transcription factor genes that have been defined in Sertoli cells are *Rhox5* and *c-myc*. *Rhox5* is androgen- and AR-induced in both cultured Sertoli cells and Sertoli cells *in vivo* (29-31, 46, 53, 62-64), while *c-myc* is androgen inducible in cultured Sertoli cells (65) but it appears not to be AR regulated *in vivo* (53, 55). In this paper, we provide several lines of evidence that we have identified 8 SAR genes that are regulated by AR through the action of *Rhox5* in the testes. First, all 8 of these genes are regulated by *Rhox5* in 15P-1 Sertoli cells (Table 1) and all but one of them (*Ifnar2*) are regulated by *Rhox5* in the testes *in vivo* (Figs. 2-4). Second, our analysis of *Ar*-deficient mice indicated that all 8 of these genes are AR regulated in adult testes *in vivo* (Fig. 5). As confirmation of this, we found that 5 of these genes (*Bpgm, Cpxm1, Ifnar2, Tmem176a,* and *Tmem176b*) were independently defined as "AR regulated" by a study that used microarray analysis to identify genes differentially expressed in postnatal *Tfm Ar*-deficient and control mice testes (55). Third, our analysis of the putative regulatory regions in the candidate SAR

genes showed that 4 of them had no consensus AREs and the other 4 had few consensus

AREs (Table 3). Because, by definition, SAR genes are regulated indirectly by AR, this

paucity of AREs is consistent with these genes being *bona fide* SAR genes. We should note,

however, that AREs are notoriously difficult to define, as consensus sites are often not

functional and it is common for functional sites to differ considerably from the consensus

sequence (66). Fourth, none of the 8 genes were regulated by AR and androgen under

conditions in which *Rhox5* was *not* induced (Supplementary Fig. 7). Fifth, a reporter

construct harboring the promoter region of one of the candidate SAR genes (*Unc5c*) was

responsive to *Rhox5*, but was not responsive to AR and androgen under conditions in which

*Rhox5* was not induced (Fig. 6). Sixth, several androgen/AR-inducible mouse *Rhox* genes

besides *Rhox5* regulated many of the candidate SAR genes (Fig. 7A). Finally, *RHOXF1*, an

androgen-inducible human *RHOX* gene (58), was also capable of regulating some of the

candidate SAR genes (Fig. 7B), providing evidence that this is a conserved response.

Interestingly, human *RHOXF2* also regulated many of these genes (Fig. 7B), but whether it is

an androgen-inducible gene has not yet been determined.

Consistent with the often noted connection between the testis and the brain (67), we

found that a remarkably large proportion of the candidate SAR genes encode proteins with

known functions in the nervous system. *Unc5c* encodes a transmembrane receptor of the

immunoglobulin superfamily that binds to netrin-1, a protein important for many

developmental events in the brain, including governing axon migration by triggering

chemorepulsion (68). UN5C also has a "death domain," which engenders it with

proapoptotic activity that may also be important for its roles in brain development (69). We

recently showed that UNC5C functions similarly in the testes by promoting the death of male germ cells (32). This germ cell death-promoting property, coupled with the fact that *Unc5c* gene expression is negatively regulated by *Rhox5*, makes *Unc5c* a good candidate to have a role in *Rhox5's* ability to promote the survival of germ cells (7). Another candidate SAR gene, *Gdap1*, is upregulated during neuron differentiation (70) and, when mutated in humans, is responsible for many cases of Charcot-Marie-Tooth disease, the most common inherited form of peripheral neuropathy. GDAP1 is a member of the glutathione-S transferase family and thus its neurologic protective role may derive from its antioxidant properties. *Tmem176b* (also known as *Clast1, Lr8,* and *TORID*) encodes a ubiquitously expressed 4-transmembrane cell-surface protein that is crucial for normal brain development and function. Targeted deletion of *Tmem176*b in mice causes defects in the cerebellum and severe ataxia (71). It has not been reported whether *Tmem176b*-mutant mice have defects in male fertility, but our finding that both *Ar* and *Rhox5* regulate its expression suggests it may have some role in androgen-mediated events in the testes. A related 4-transmembrane protein-encoding gene, *Tmem176a* (also known as *GS188* and *HCA112*), is another putative SAR gene. At present, there is no information on either the biochemical or biological function of *Tmem176a*. Another candidate SAR gene, *Pltp*, encodes a protein that acts both inside and outside of cells to mediate the transfer of lipids. While PLTP is fairly widely expressed and therefore probably acts in many cell types, it appears to be particularly important for brain function, in part, by virtue of its being secreted by neurons and glial cells. PLTP is likely to have roles in neurodegenerative and inflammatory brain diseases, including Alzheimer's disease, as PLTP levels are altered in many of these disease states. PLTP also alters the

phosphorylation state of TAU, a neuron-specific protein that undergoes shifts in phosphorylation status in many neurological diseases (72).

The remaining three candidate SAR genes, *Fzd2, Ifnr2*, and *Bpgm*, are likely to have roles in many cell types. *Fzd2*, encodes 1 of the 10 known frizzled receptors in mice; these are cell-surface receptors that bind to the WNT family of secreted glycoproteins. Upon binding of WNT proteins, Frizzled receptors elicit signal transduction events crucial for conserved developmental steps in many organisms. Because frizzled receptors tend to act redundantly, it has been difficult to assign precise functions to them, including FZD2 (73). *Ifnar2* encodes the cell surface receptor for anti-viral cytokines called interferons. Upon interferon binding, IFNAR2 activates the STAT signaling pathway, which in turn elicits an anti-viral response, including rapid mRNA decay and translational repression (74). *Bpgm* encodes a multifunctional enzyme best known for its role in controlling the metabolism of 2,3-diphosphoglycerate in erythrocytes (75). It will be interesting to know what specific roles BPGM has in the testes.

In addition to the candidate SAR genes described above, we identified 4 other genes regulated by *Rhox5* in the testes *in vivo: Cd24a, Klf9, Cpxm1,* and *Tmem47* (Figs. 2-4). Two of these, *Cd24a* and *Klf9*, also have known roles in the nervous system. *Cd24a* encodes a glycosylphophatidylinositol-anchored glycoprotein that was originally identified on the basis of its expression in immune cells but was subsequently shown to also be expressed and have a role in neurons. In the adult mouse central nervous system, CD24 expression is restricted to immature proliferating neurons in regions of the brain undergoing neurogenesis. CD24 serves to dampen the proliferation of these neurons, as *Cd24a*-knockout mice exhibit

increased numbers of proliferating neurons in these zones (76). *Kfl9* (also known as *Bteb1*) encodes a zinc-finger transcription factor that when knocked out in mice leads to cerebellar Purkinje cell defects and behavioral defects consistent with its having roles in the cerebellum, amygdala, and hippocampus (77). While exactly how KLF9 functions in the brain is not known, *in vitro* experiments have suggested it has a role in neurite extension and branching *in vitro* (44). *Tmem47* encodes a 4-transmembrane protein that is a member of a different subfamily than *Tmem176a* and *Tmem176b*, the other 4-transmembrane protein genes regulated by *Rhox5*. The functional role of TMEM47 in mammals is not known, but its ortholog in *C. elegans,* VAB-9, is crucial for the formation of adherens junctions in epithelial cells (78). Thus, it is tempting to speculate that TMEM47 might function in the formation and/or maintenance of the testes-specific adherens junctions that form between Sertoli cells and germ cells: the ectoplasmic specialization.

Future studies will be required to precisely define the gene networks downstream of AR and the AR-regulated *Rhox* genes and to identify their roles in spermatogenesis. One essential question will be to delineate which functions of AR require the action of androgen-regulated *Rhox* genes. Studies in AR-deficient mice harboring either a non-functional *Ar* allele in Sertoli cells or a hypomorphic *Ar* allele in all cells have revealed that AR has at least 3 functions: (i) it promotes the progression of meiotic spermatocytes, (ii) it promotes the maturation and/or survival of round spermatids, allowing the generation of elongating spermatids, and (iii) it promotes the final maturation and release of elongated spermatids into the lumen of the seminiferous tubules (62, 64, 79, 80). Thus, androgen-inducible *Rhox* genes may be involved in any or all of these events. Another question for the future will be

to determine which *Rhox5*-regulated genes in Sertoli cells are direct targets of RHOX5.   This

will be a challenge to answer, as a RHOX5-binding consensus sequence has yet to be defined

and RHOX5 may act on enhancers far upstream or downstream of its target genes.   Finally,

it will be intriguing to understand how *Rhox5* regulates genes in cell types in addition to the

Sertoli cell, the only cell type that significantly expresses *Rhox5* (26, 29).   Given that a large

proportion of *Rhox5*-regulated genes encode cell-surface and secreted proteins (Supplemental

Fig. S3), an obvious mechanism by which it could act is to influence cell-cell interactions and

signaling events occurring between Sertoli cells and other cell types in the testis.   By

defining and characterizing *Rhox*-regulated genes *in vitro* and *in vivo*, we believe that the

work presented in this paper provides a foundation to address these many future issues.

**Materials and Methods**

**Cell Culture and Transfection**

The 15P-1 and MSC1 cell lines were maintained in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum and 50 mg/ml of both penicillin and streptomycin.

The cells were grown at $37^{o}$C in 5% $CO_2$ atmosphere and split when ~80% confluent.

To generate stable *Rhox5* expression plasmid transfected clones, 15P-1 cells cultured to ~60%

confluency in 100 mm plates were transfected with 2 μg of *Rhox5* expression plasmid using

lipofectamine (Invitrogen, Carlsbad, CA) and clones resistant to antibiotic (700 μg/ml G418)

were selected.

The concentration of plasmids used for transfection was independently determined using both a fluorometer and analytical gel electrophoresis. For transient transfection analysis, 15P-1 cells were plated in 60mm dishes the day before transfection at a density of ~$2\times10^6$ cells per dishes in 4 ml of growing medium. On the day of transfection, 800 ng of plasmid DNA was diluted with 150 μl of serum-free medium and mixed with 150 μl of serum-free media containing 3 μl Lipofectamine (Invitrogen, Carlsbad, CA), incubated at room temperature for 30 min, and the DNA-lipid complex was added to the wells and incubated at 37 °C in a $CO_2$ incubator for 5 h. Cells were harvested 36 h post-transfection for RNA analysis. MSC1 cells were transfected using Fugene 6 (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's instructions. Cells were plated on 12-well culture dishes and then cotransfected with 100 ng of the *Rhox5*/*Unc5c*-containing plasmids and 100 ng of either the AR expression plasmid or empty vector. After a 6-h incubation in DMEM supplemented with 10% charcoal-stripped bovine serum (HyClone Laboratories, Inc., Logan, UT), R1881 was added at final concentration of 10 nM. Cells were harvested 36 h post-transfection for luciferase assay using the Dual Luciferase Assay System (Promega, Madison, WI), following the manufacturer's instructions. The relative luciferase activity was calculated by normalizing against the co-transfected internal control pRL-null. The results shown are the mean ± SEM of three independent transfection experiments.

**Plasmids**

The *Unc5c*-P (G734) and *Rhox5*-Pp (Pem250) plasmids used in this study were generated previously (32, 46). The Rhox expression plasmids used in this study were generated previously (32). The human AR pcDNA 3.1 plasmid (G541) was kindly provided by Zhengxin Wang (The University of Texas M. D. Anderson Cancer Center, Houston, TX).

**RNA and protein analysis**

Total cellular RNA was prepared from cell lines and tissues as described previously by guanidinium isothiocynate lysis and centrifugation over a 5.7 M CsCl cushion (81). The *Rhox5* cDNA probe used for Northern blot analysis was prepared as described previously (24). Real-time reverse transcriptase (RT) polymerase chain reaction (PCR) analysis was performed as described previously (7) using primers as described in Table 1. Microarray analysis with 15P-1 stable cell clones was performed at the University of Iowa DNA core facitlity on total cellular RNA from clones no.6, 24, 14, and 16 (refer to Fig. 1C), using an Affymetrix mouse genome GeneChip 430 2.0 array. This gene chip independently samples approximately 45,101 probe sets, which corresponds to approximately 39,000 genes.

For western blot analysis, 20 μg of total cell lysates were electrophoresed in a 10% SDS–polyacrylamide, transferred to Hybond ECL nitrocellulose (Amersham, Piscataway, NJ), and probed with antibody against RHOX5 or β-actin (Sigma, St. Louis, MO). The membranes were given three 10-min washes with 0.1% Tween-20 in phosphate-buffered saline at room temperature and then incubated for 45 min at room temperature with the 2° antibody (ECL kit anti-rabbit or anti-mouse from Amersham, Piscataway, NJ) at a dilution of 1:5,000. The

filter was developed by using the ECL-Plus reagent according to manufacturer's protocol (Amersham, Piscataway, NJ).

**Testis cell fractionation**

Sertoli and interstitial cells were purified from testes as previously described (7, 82). In brief, testes were decapsulated and the seminiferous tubules were allowed to settle in phosphate-buffered saline, followed by incubation in collagenase (C2674; Sigma Inc.). Following another round of settling, the supernatant, which was enriched for interstitial cells, was subjected to hypotonic shock (by incubation in 1:7 diluted phosphate-buffered saline for 3 min) to remove germ cells, and the surviving cells were pelleted and the RNA extracted. The pellet obtained after collagenase treatment, which was enriched for Sertoli cells, was incubated in a solution containing a mixture of enzymes (0.1% collagenase, 0.2% hyaluronidase [H6254; Sigma], 0.04% DNase I [D5025；Sigma], and 0.03% trypsin inhibitor [T6522; Sigma] in 1×PBS, pH7.4) at 30 °C for 40 min. The cells were pelleted and subjected to the same hypotonic shock as the interstitial cells, followed by centrifugation and RNA extraction of the pellet.

**Figure Legends**

**Fig. 1.** Generation of 15P-1 Sertoli cell clones expressing physiological levels of *Rhox5*. A, Western blot analysis of RHOX5 protein expression in 15P-1 stable clones stably transfected with a RHOX5-expression vector. SL12.4 cells served as positive control for RHOX5 expression. Following probing with RHOX5 antiserum, the blot was stripped and reprobed with a β-actin antiserum as an internal control for loading. B, qPCR analysis of total cellular RNA from the 15P-1 Sertoli cell clones. *Rhox5* mRNA expression levels in the 15P-1 cell clones relative to parental 15P-1 cells (which was arbitrarily given a value of "1"), all normalized against ribosomal protein L19 transcript levels. Adult testis served as a positive control. Values denote the mean fold change ± standard error of mean (SEM).

**Fig. 2.** Genes regulated by *Rhox5* in the adult testis. qPCR analysis of total cellular RNA from adult *Rhox5*-null (*KO*) and wild-type (*WT*) littermate testes. mRNA levels were quantified as described in Fig. 1B. Statistical analysis was performed using the two-tailed Student's *t* test: *, $p<0.05$; **, $p<0.01$.

**Fig. 3.** Cell subset expression pattern of *Rhox5*-regulated genes. qPCR analysis of total cellular RNA from purified Sertoli and interstitial cells (prepared using hypotonic shock to remove germ cells) from adult *Rhox5*-null (*KO*) and wild-type (*WT*) littermate mice testes. The value shown for each gene is the average WT/KO mRNA ratio from two independent cell preparations, quantified as described in Fig. 1B. a, mRNA ratios of either $\geq 2$ or $\leq 0.5$ (i.e., >2-fold change).

**Fig. 4.** Developmental expression pattern of *Rhox5*-regulated genes. qPCR analysis of testes RNA from *Rhox5*-null (*KO*) and wild-type (*WT*) littermate mice of the indicated postnatal ages (6 mice per time point). All values were quantified as described in Fig. 1B. Postnatal time points at which *Rhox5* regulates the indicated genes are denoted by arrows (upward and downward arrows indicate genes that are upregulated and downregulated, respectively). A value of "1" was arbitrarily given to the lowest expression level among the time points examined for a given gene. Error bars indicate standard error.

**Fig. 5.** Identification of *Ar*- and *Rhox5*-regulated genes. A, qPCR analysis of testes RNA from adult *Tfm* and littermate control mice. B, qPCR analysis of testes RNA from adult *Scarko* and littermate control mice. All values were quantified as described in Fig. 1B. Control mRNA levels were arbitrarily given a value of "1." Statistical analysis was performed using the two-tailed Student's *t* test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

**Fig. 6.** The candidate SAR gene *Unc5c* responds to *Rhox5* but not *Ar* and androgen *in vitro*. A, Schematic diagram of *Unc5c* promoter and *Rhox5* proximal promoter (*Pp*) constructs harboring the firefly luciferase (Luc) reporter. B, Luc expression from MSC1 cells transiently cotransfected with *pGL3-Unc5c* (100 ng), a Renilla luciferase vector (pRL-Tk) (25 ng) for an internal control, and either a *Rhox5*-expression vector or the corresponding empty expression vector (300 ng). C, Luc expression from MSC1 cells transiently transfected with the constructs shown in panel A (100 ng) and pRL-Tk (25 ng). Where indicated with

"+AR," the cells were cotransfected with an AR expression vector (100 ng). Where indicated with "+T," the cells were incubated with the synthetic androgen R1881. The relative Luc activities shown are relative to non-treated cells (which were arbitrarily given a value of "1") from three independent transfection experiments. Error bars indicate standard error. Note that AR + T did not induce endogenous *Rhox5* expression in these cells (data not shown), explaining why *Unc5c* expression was not regulated.

**Fig. 7.** Regulation of candidate SAR genes by *Ar*-inducible *Rhox* genes. A, qPCR analysis of total cellular RNA from 15P-1 Sertoli cells transiently transfected with expression vectors encoding the indicated mouse *Rhox* genes or the empty expression vector (800 ng). A value of "1" was arbitrarily given to the expression of the indicated genes in cells transfected with the empty expression vector. All values were quantified as described in Fig. 1B. B, Same as panel A, except the cells were transiently transfected with expression vectors encoding the human RHOXF1 and RHOXF2 proteins.

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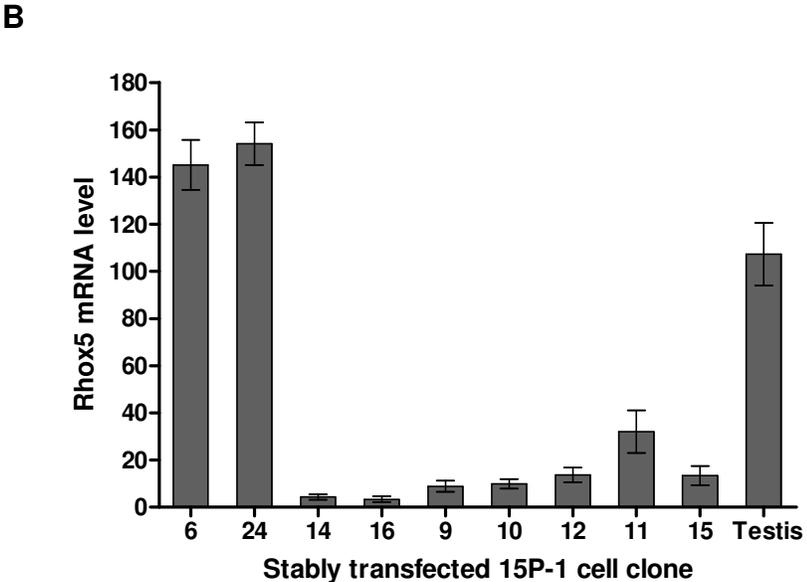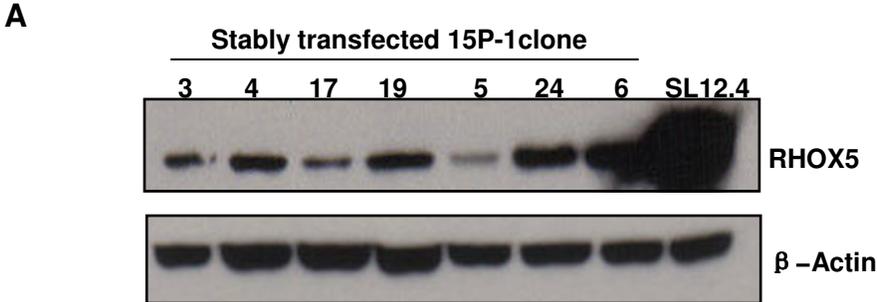
# Figure 1

**A**

Stably transfected 15P-1clone



**B**

**Table 1. *Rhox5*-Regulated Genes**

| Gene Symbol | Gene name | Array | qPCR | Molecular function | Celluar component |
|---|---|---|---|---|---|
| Tmem47 | Transmembrane protein 47 | 253 | 519 | Signal transduction | Membrane |
| Cd24a | CD24a antigen | 16.6 | 15.6 | Apoptosis | Membrane |
| Gdap1 | Ganglioside-induced differentiation associated protein 1 | 10.5 | 4.0 | Signal transduction | Cytoplasm |
| Tfrc | Transferrin receptor | 8.5 | 2.8 | Transport | Membrane |
| Bpgm | 2,3-bisphosphoglycerate mutase | 3.0 | 2.7 | Metabolism | Unknown |
| Idh3a | Isocitrate Dehydrogenase 3 | 2.6 | 5.0 | Metabolism | Mitochondrion |
| Fzd2 | Frizzled homolog 2 | -2.1 | -2.7 | Signal transduction | Membrane |
| Tmem176b | Transmembrane protein 176B | -2.1 | -1.6 | Signal transduction | Membrane |
| Tmem176a | Transmembrane protein 176A | -2.2 | -2.2 | Unknown | Membrane |
| Bach2 | BTB and CNC Homology 2 | -2.6 | -6.6 | Transcription factor | Nucleus |
| Pltp | Phospholipid transfer protein | -3.2 | -5.0 | Transport | Membrane |
| Klf9 | Kruppel-like factor 9 | -3.2 | -2.3 | Signal transduction | Nucleus |
| Ifnar2 | Interferon (alpha and beta) receptor 2 | -3.5 | -3.2 | Signal transduction | Membrane |
| Unc5c | Unc5 homolog C | -3.5 | -9.5 | Signal transduction | Membrane |
| Cpxm1 | Carboxypeptidase X1 | -8.1 | -10.3 | Transport | Extracellular |

The "Array" column indicates the average change in mRNA level in the *Rhox5*-positive expressing 15P-1 Sertoli cell clones (#6 and #24) relative to the *Rhox5*-negative cell clones (#14 and #16), as judged by microarray analysis. The "qPCR" column lists the average mRNA levels of the same genes, as judged using qPCR analysis, normalized against the level of L19 mRNA (see Fig. 1B). Positive and negative values denote gene expression that was upregulated and downregulated, respectively, in response to *Rhox5*. The genes were classified into "molecular function" and "cellular component" using OntoExpress software.

**Table 2. Cell types expressing *Rhox5* - regulated genes**

|  | Gene | Sertoli cells | Interstitial cells | Sertoli No-shock | Interstitial No-shock | Primary Source |
|---|---|---|---|---|---|---|
| Group 1 | *Gdap1* | 2.2 | 1.0 | 1.2 | 1.6 | Sertoli |
|  | *Tfrc* | 1.9 | 0.2 | 0.7 | 0.5 | Sertoli |
|  | *Tmem176b* | 2.7 | 0.4 | *0.2* | 0.3 | Sertoli |
|  | *Tmem176a* | 2.8 | 0.8 | *0.3* | 0.3 | Sertoli |
|  | *Ifnar2* | 8.3 | 0.9 | 0.4 | 1.4 | Sertoli |
| Group 2 | *Tmem47* | 8.9 | 2.2 | 2.7 | 0.1 | Sertoli +Interstitial |
|  | *Fzd2* | 8.3 | 6.3 | 1.7 | 0.5 | Sertoli +Interstitial |
|  | *Pltp* | 5.5 | 4.3 | 0.9 | 1.6 | Sertoli +Interstitial |
|  | *Unc5c* | 1.9 | 2.0 | 1.1 | 1.4 | Sertoli+Interstitial |
| Group 3 | *Cd24a* | 0.7 | 7.7 | 0.7 | 1.3 | Interstitial |
| Group 4 | *Bpgm* | 0.4 | 0.7 | 0.1 | 0.4 | Non-Sertoli/Interstitial |
|  | *Idh3a* | 0.9 | 0.1 | 1.1 | 1.1 | Non-Sertoli/Interstitial |
|  | *Bach2* | 0.5 | 0.4 | 0.8 | 1.1 | Non-Sertoli/Interstitial |
|  | *Klf9* | 0.8 | 0.7 | 1.1 | 0.9 | Non-Sertoli/Interstitial |
|  | *Cpxm1* | 0.2 | 0.04 | 0.4 | 0.9 | Non-Sertoli/Interstitial |
| Markers | *Gata1* | 4.2 | 0.1 | 3.8 | 0.1 | Sertoli |
|  | *Rhox5* | 5.3 | 0.9 | 1.5 | 0.2 | Sertoli |
|  | *Lhr* | 0.6 | 19.9 | 0.2 | 1.0 | Interstitial |
|  | *Tr2* | 0.7 | 0.2 | 1.0 | 1.9 | Germ |
|  | *Dazl* | 0.5 | 0.1 | 2.2 | 1.0 | Germ |

qPCR analysis of total cellular RNA from purified Sertoli and interstitial cells obtained from adult testes. The "no shock" columns refer to the semi-purified Sertoli and interstitial cell fractions that had not undergone hypotonic shock to remove most germ cells. The numbers are mRNA level (normalized against L19 mRNA; see Fig. 1B) in the cell fractions relative to total testes, the latter of which was arbitrarily given a value of "1."
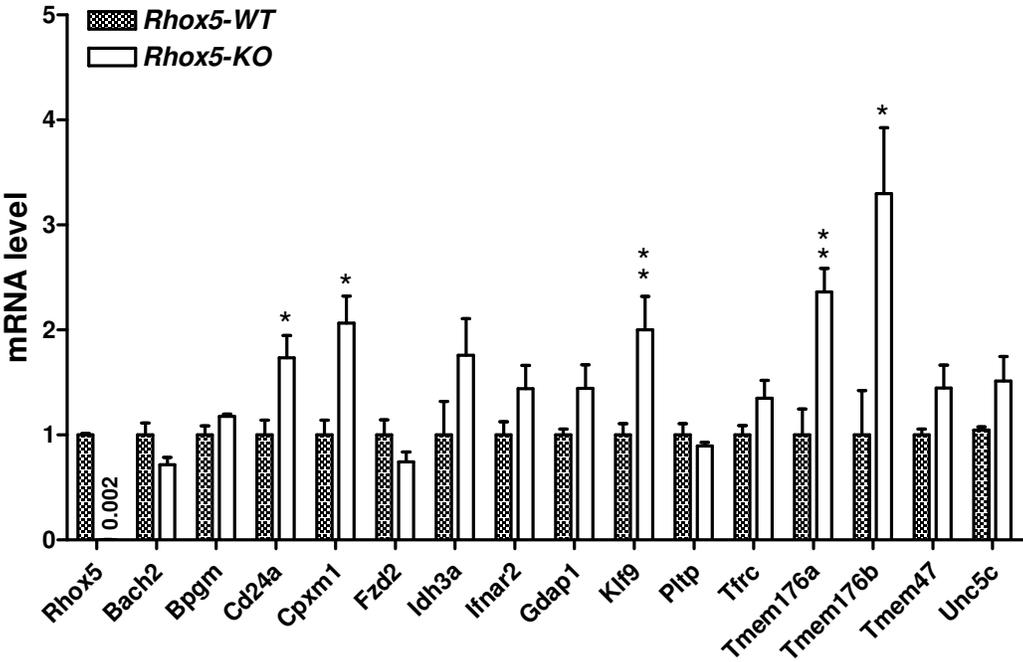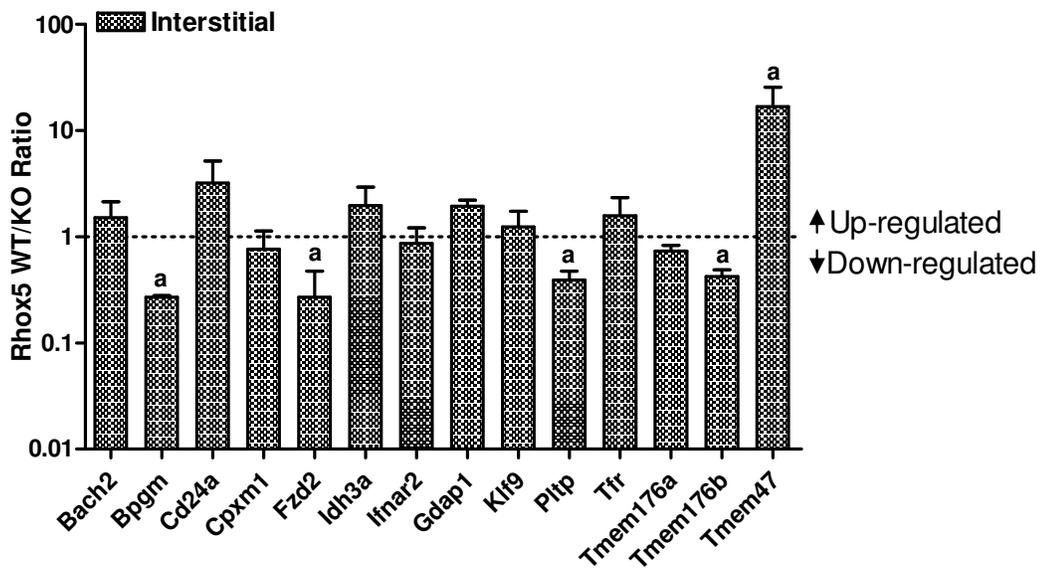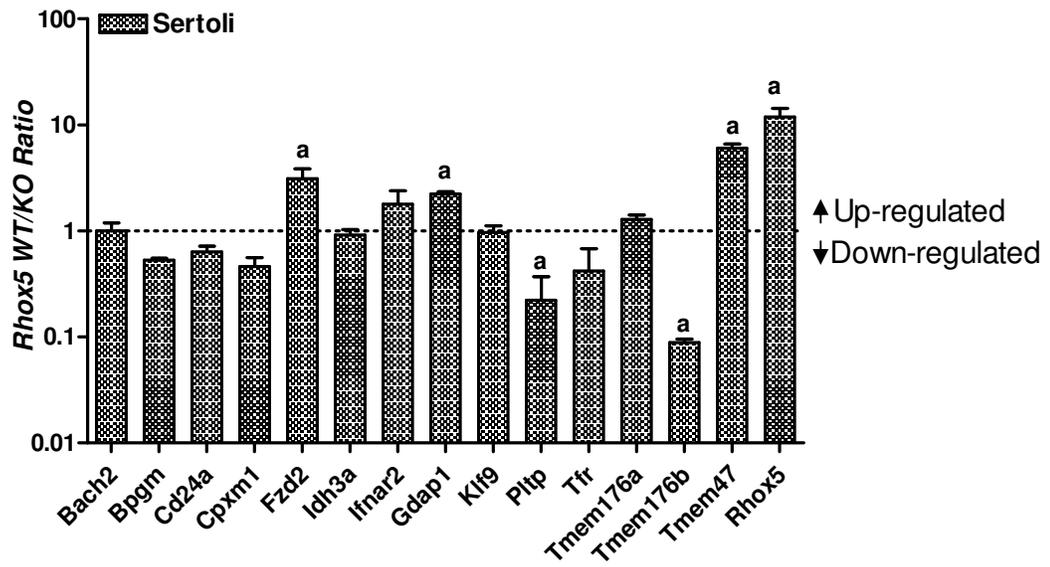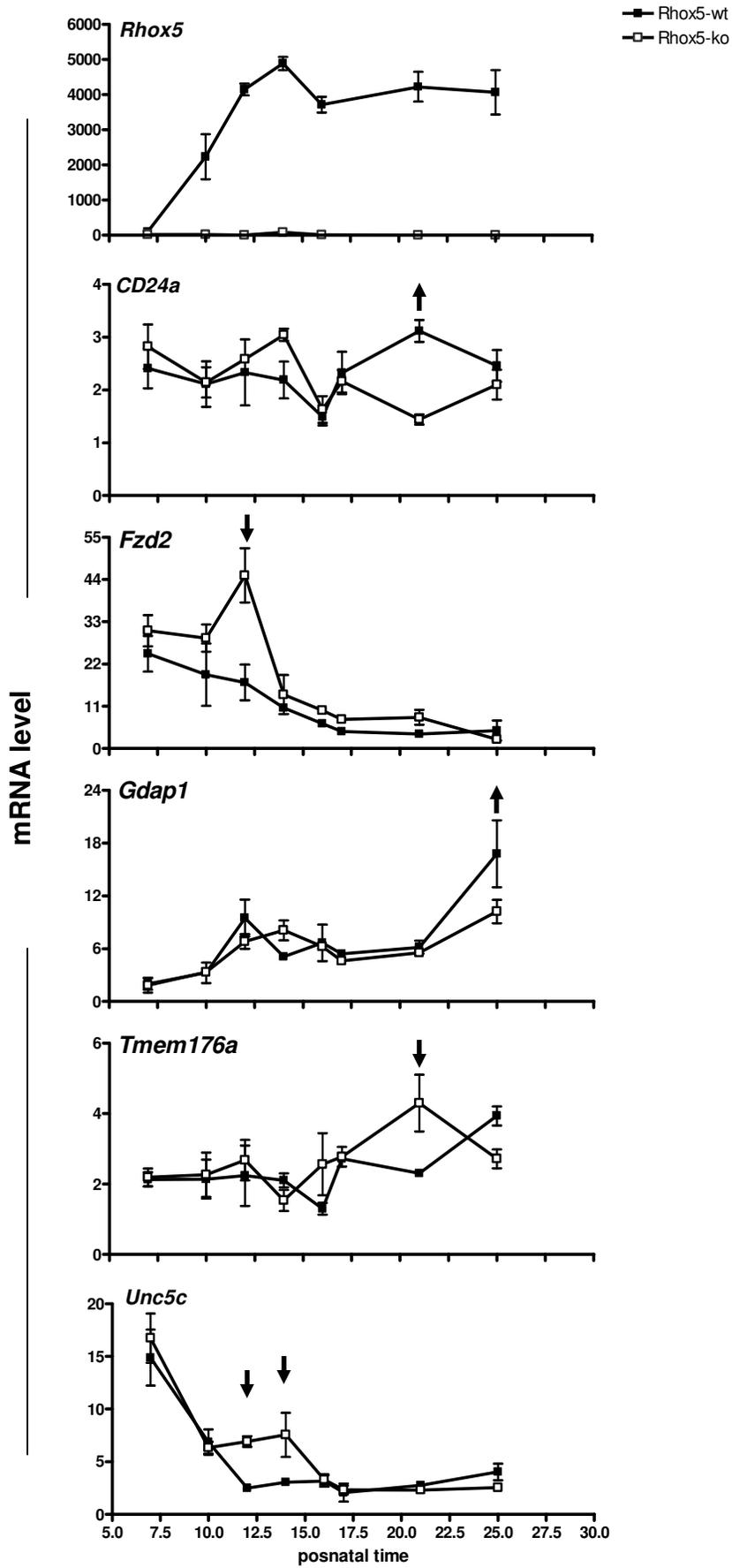
**Figure 2**

**Figure 3**

**Figure 4**

**Figure 5**
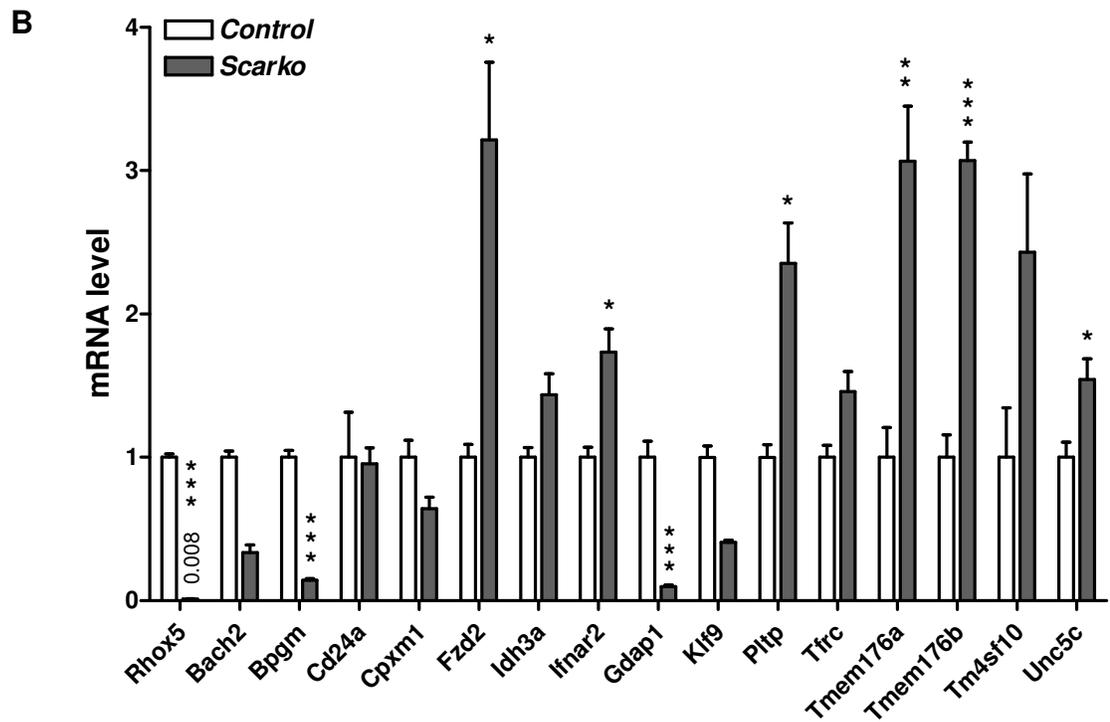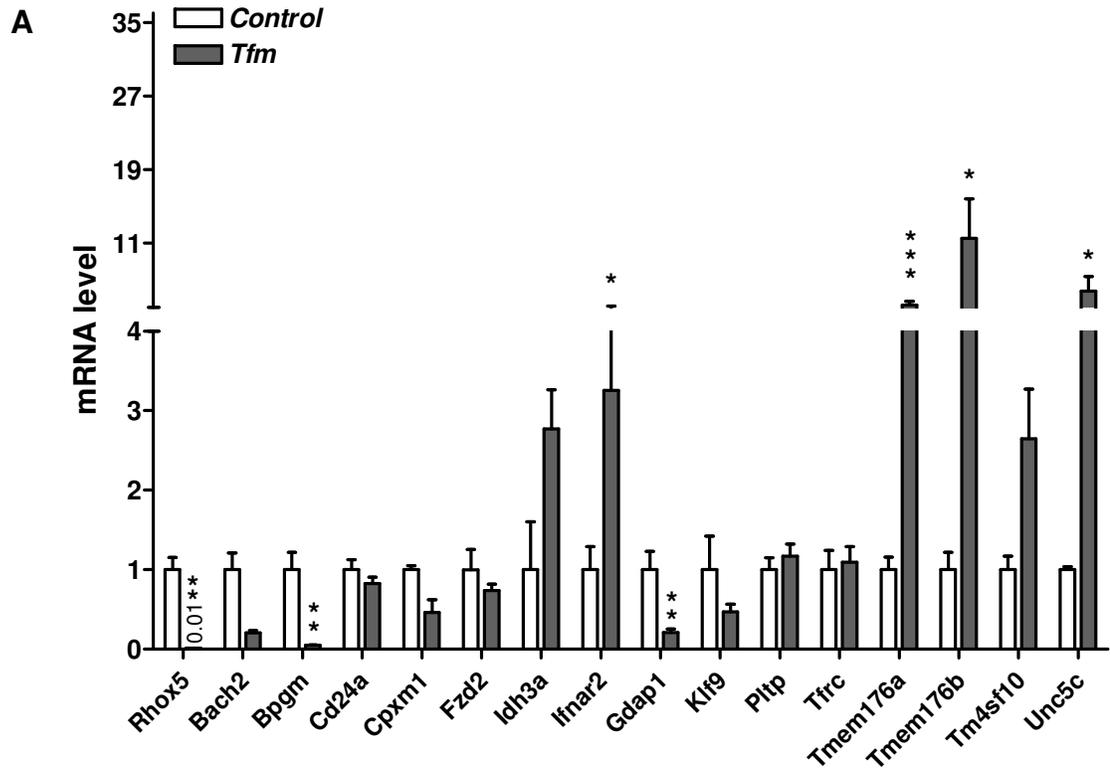
**Figure 6**

**Figure 7A.**

**Figure 7B.**