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Mice with Y chromosome deletion and reduced *Rbm* genes on a heterozygous *Dazl1* null background mimic a human azoospermic factor phenotype

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A subset of azoospermia or oligozoospermia patients have microdeletions in defined regions of their Y chromosome, namely the AZFa, b, and c regions. Candidate genes in humans that may cause the azoospermia factor (AZF) phenotype have been assigned to these regions and can include the *DAZ* and *RBM* genes. Part of the variability in the AZFc phenotype might be due to interaction between the effects of deleting the *DAZ* and *RBM* genes. We mimicked human deletions of *RBM* and *DAZ* in the mouse by crossing male mice with a deleted Y chromosome with a reduced number of *Rbm* genes (Y^{d1}) to heterozygote *Dazl1* null female mice to study the interaction of the *Dazl1* and *Rbm* or other genes located in the Y^{d1} deletion interval. *Dazl–/+* Y^{d1} animals showed a significant reduction in the sperm count ($P < 0.001$), an increase of abnormal sperm heads and prominent mid-piece defects of the tails compared to either mutation alone ($P < 0.001$). Hence, *Dazl1* and the genes removed on the Y^{d1} chromosome are active in different pathways contributing to different stages of spermatogenesis. Reduction of *Dazl1* and *Rbm* genes as well as/or deletion of the Y chromosome in mice gives rise to a phenotype similar to the heterogeneous AZFc phenotype observed in humans.

Key words: AZF/*Dazl1*/*Rbm*/spermatogenesis/Y chromosome

Introduction

The human Y chromosome can be divided into recombining and non-recombining parts: the pseudo-autosomal regions contain genes with homologues on the X chromosome and the remainder which is excluded from recombination and carries genes which are mostly male specific. While the former genes are mainly ubiquitously expressed, many genes localized in the non-recombining region are restricted to a testis specific expression and these are considered to serve a role exclusively in testis determination and male germ cell development (Lahn and Page, 1997). These genes are scattered over the euchromatic regions on both of the Y chromosome's arms but particularly deletions in Yq11 are relatively frequent in the human population and are often associated with infertility through non-obstructive azoospermia or severe oligozoospermia (reviewed by Vogt, 1998; Krausz and McElreavey, 1999). It was therefore initially postulated that an azoospermia factor (AZF) was localized in this region (Tiepolo and Zuffardi, 1976) and through recent refinement of molecular techniques and markers in Yq11 the AZF has been subdivided into at least three non-overlapping regions, designated as AZFa, b, c (Vogt *et al.*, 1996). About 13% of men with non-obstructive azoospermia have microdeletions in the AZFc region, deletions in AZFa and b are less frequent (summarized in Vogt, 1998; Krausz and McElreavey, 1999). Testis biopsies of a small cohort of patients with azoo- or oligozoospermia have been investigated and different histological phenotypes have been associated with deletions of AZF intervals (Vogt *et al.*, 1996). These authors suggest that deletions in the AZFa region are associated with Sertoli cell only syndrome, AZFb deletions with a premeiotic maturation arrest and AZFc phenotypes with a heterogeneous phenotype including Sertoli cell only syndrome as well as pre- and postmeiotic maturation defects. Results from different studies are not consistent with a direct link between phenotype and deletion interval (Pryor *et al.*, 1997), and histology cannot predict the particular Y deletion present.

Several genes have been mapped into each of the AZF intervals (Lahn and Page, 1997) but for most of them their individual contribution to the AZF phenotype remains to be determined. However, biochemical characterization of some gene products and functional studies of different animal homologues led to the assignment of at least one strong candidate gene for each microdeletion interval, namely *DFFRY* (Brown *et al.*, 1998) for AZFa, *RBM* (Ma *et al.*, 1993; Elliott *et al.*, 1997) for AZFb, and *DAZ* (Reijo *et al.*, 1995) for AZFc, all of which may cause azoospermia if deleted.

Despite a growing number of screening programmes for deletions in infertile men involving the AZF regions, the link between *RBM* and *DAZ* deletions and impaired spermatogenesis is still formally unproven. All microdeletions described so far encompass a substantial amount of DNA and there is evidence that genes other than *RBM* or *DAZ* might also be affected by the deletions (Lahn and Page, 1997; Wong *et al.*, 1999). Moreover, deletions within the AZFc region that apparently do not include the *DAZ* gene family have been reported in azoospermic individuals (Najmabadi *et al.*, 1996; Stuppia *et al.*, 1997; Foresta *et al.*, 1997).

Proof of a causative link between *RBM* and *DAZ* genes and impaired spermatogenesis would be the detection of point mutations, small intragenic deletions or rearrangements in these genes, which should give rise to the same phenotype as observed in men with microdeletions of Yq11. This goal has not been achieved to date but is of importance as concerns arise about the functionality of the *DAZ* genes in general (Vereb *et al.*, 1997; Agulnik *et al.*, 1998).

Table I. Categories and numbers counted of normal and abnormal sperm heads. *n* = total number of sperm heads counted. The *Dazl*^{Tm1Hgu} allele is referred to as –, the wild type *Dazl1* allele as +; wt = wild type.

Table 1

Nr. genotype	normal N	%	slightly abnormal 1	1a	1b	1c	1d	%	grossly abnormal 2	3	4	5	6	%	n
1 +/+ Y^{wt}	195	97.5 ±6.2				3		1.5 ±2.5		1	1			1	200
2 +/- Y^{wt}	180	88.83 ±3.6	2		7			5.33 ±1.5	1	4	2	1	3	5.83	200
3 +/- Y^{wt}	173		1	5	1	7	1			4	1	1	6		200
4 +/- Y^{wt}	180		2		5	1				4	2	3	3		200
5 +/+ Y^{d1}	124	69.17 ±3.6		16	15	24		23.33 ±1.5		11	10			7.5	200
6 +/+ Y^{d1}	149		4	15	11	11			4	4	2				200
7 +/+ Y^{d1}	142		1	13	17	8	5		1	9	3	1			200
8 +/- Y^{d1}	67	26.25 ±3.1	15	11	8	15	9	28.75 ±1.3	6	18	37	7	7	45	200
9 +/- Y^{d1}	35		12	3	6	21	10		3	22	70	13	5		200
10 +/- Y^{d1}	44		7	7	4	16	24		3	19	64	8	5		200
11 +/- Y^{d1}	64		7	18	3	16	18		6	13	48	5	2		200

Figure 1

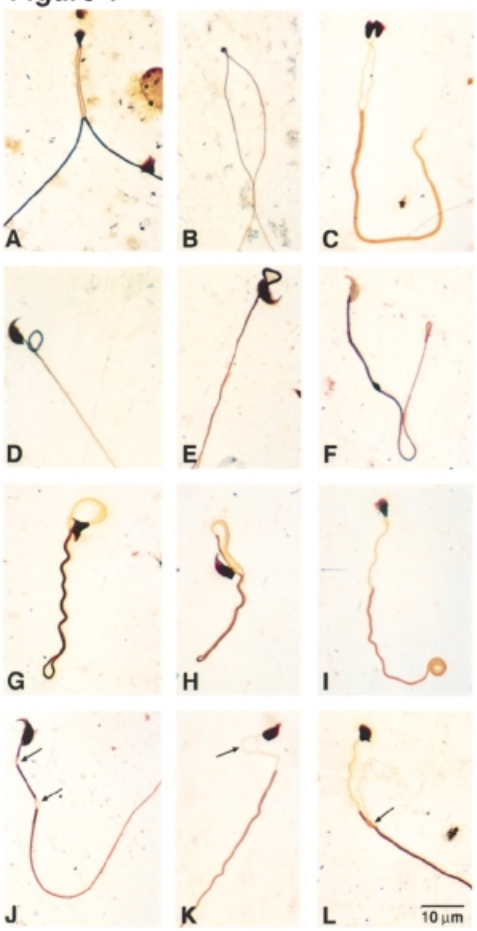
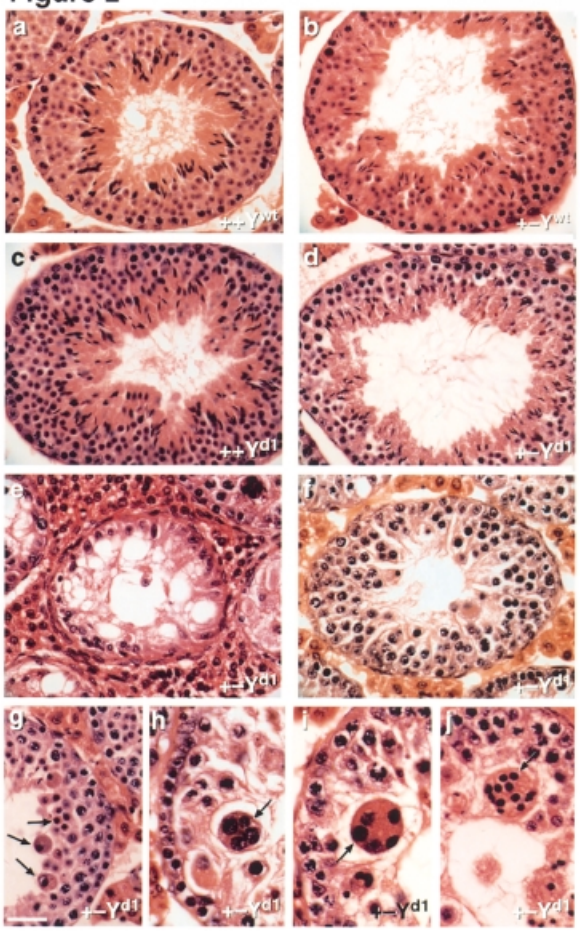


Figure 2



The individual features of these genes have hampered the investigation of the controversial role of *RBM* and *DAZ* as AZF: *RBM* is a multigene family of at least 20–40 micro-heterogeneous members on the Y chromosome. While a minimal critical region for RBM expression in AZFb has been determined (Elliott *et al.*, 1997), it is difficult to assess whether deletions or sequence variations affect 'the' essential gene/genes.

The situation for *DAZ* is even more complicated as the micro-heterogeneous multicopy organization on the Y comes together with a highly related homologous gene on chromosome 3, *DAZL1* (Saxena *et al.*, 1996), whose function in relation to *DAZ* is poorly understood.

Two approaches have been used to make genotype-phenotype correlations of the candidate genes for AZF: one is to investigate the biochemical properties of RBM and DAZ, which both seem to be involved in RNA metabolism (Houston *et al.*, 1998; Elliott *et al.*, 1998), the other is to address this question in model organisms that are more accessible to study defined alterations of the genes. *DAZ* homologues of *Drosophila* (*boule*) (Eberhart *et al.*, 1996), *Xenopus* (*Xdazl*) (Houston *et al.*, 1998), and *C. elegans* (*dazl1*) (Karashima *et al.*, 1997) have all been shown to function in male and/or female germline-specific processes. The mouse is, however, the only animal model in which both genes have been investigated. The conserved Y chromosomal location of *Rbm* (Elliott *et al.*, 1996) has hampered a simple knock-out experiment, but asymmetrical pairing and exchange of Yp-Sxr^a has produced a sex reversed mouse line with defined deletions of the 25–50 *Rbm* copies (Capel *et al.*, 1993; Laval *et al.*, 1995). In one of these lines, namely Y^{d1}, the number of residual *Rbm* genes was estimated as 2–4. The introduction of an *Sry* transgene into this line produced males with only low level transcription of *Rbm* paired with an increased proportion of abnormal sperm heads. The sperm count and the overall fertility of these animals was unaffected (Mahadevaiah *et al.*, 1998). These authors attribute the observed sperm head abnormalities to the lack of *Rbm* function. However, since the deletion interval of the Y^{d1} chromosome is estimated to encompass 3–4 Mb, disruption of genes other than *Rbm* may also be involved in the Y^{d1} phenotype.

The human *DAZ* genes are a recent acquisition to the Y chromosome and are only found in humans and old world monkeys. The murine homologue *Dazl1* (Reijo *et al.*, 1996; Cooke *et al.*, 1996) however is located on chromosome 17, and readily accessible for knock-out experiments. Heterozygote *Dazl^{Tm1Hgu}*/+ animals have a reduced sperm count and show minor sperm tail defects, but are fertile. The reproductive organs of homozygous males and females are devoid of meiotic germ cells and *Dazl^{Tm1Hgu}/Dazl^{Tm1Hgu}* animals are therefore infertile (Ruggiu *et al.*, 1997).

Since both *DAZ* and *RBM* are multigene families in man and since deletions of the Y chromosome can include both *DAZ* and *RBM* genes simultaneously and to varying extents (Pryor *et al.*, 1997; Elliott *et al.*, 1997), we reasoned that a part of the variability in phenotype might be due to interaction between the effects of deleting the two genes and/or other genes located in the vicinity. To mimic this in the mouse we crossed the Y^{d1} chromosome on a *Dazl^{Tm1Hgu}*/+ background to investigate the resulting phenotype. Since a Y chromosomal localization of *RBM* is conserved in mammals, it is likely that genes interspersing the *RBM* family in humans might also be found in similar location in mouse. Hence, although the Y^{d1} deletion is not fully characterized with respect to affected genes other than *Rbm*, the same genes might be removed by human AZF deletions. If Dazl1 and Rbm (or other gene products) are acting in different molecular pathways these mutations might be expected to have additive or synergistic effects which might be expected to lead to infertility, whereas if acting in the same pathway the effect of combining the mutations might be minimal.

Materials and methods

Mice

The control and *Dazl^{Tm1Hgu}*/+ mice were maintained on a random bred MF1 (OLAC) background. The Y^{d1} males were produced as previously described (Capel *et al.*, 1993; Laval *et al.*, 1995) by BMC, at the MRC Radiobiology Unit. The *Dazl^{Tm1Hgu}*/+ *Y^{d1}* males were produced by crossing male Y^{d1} and female *Dazl^{Tm1Hgu}*/+. The control animal was 9 weeks, all other males investigated were 13–14 weeks of age.

Sperm analysis

Sperm counts per epididymis were carried out as previously described (Searle and Beechey, 1974). Spermatozoa for morphology analysis were released from the epididymis into phosphate-buffered saline, the suspension smeared onto clean slides and allowed to dry. Slides were then fixed in methanol for 10 min at room temperature (RT) and air-dried. Preparations were silver-stained (Howell and Black,

Figure 1. Tail defects. (**A**) Double tails, the mid-piece region still conjoined. (**B**) Micro-headed spermatozoa with two completely separated tails. (**C**) Double mid-piece with two heads. (**D–F**) Abnormalities most frequently seen in *Dazl^{Tm1Hgu}*/+ males. (**D** and **E**) Coiled mid-piece. (**F**) Kinked flagellum and tail tip. (**G–L**) Abnormalities only seen in *Dazl^{Tm1Hgu}/ Y^{d1}* males. (**G** and **H**) Tails coiled and self-adhering. (**I**) Catherine-wheel tail tip. (**J**) Small regions of breakdown (arrows) in both mid-piece and flagellum. (**K**) Mid-piece split into two subsections. (**L**) Small region of flagellum disrupted, with fibre extrusion. Bar = 10 μm.

Figure 2. Histology. (**a–d**) Sections at stage VII of spermatogenesis. (**a**) +/+ Y^{wt}, normal with all germ cell types present. (**b**) *Dazl^{Tm1Hgu}*/+, all germ cell types present, but regions showing reduced round spermatids and absent elongating spermatids. Tubule lumen larger than normal. (**c**) +/+Y^{d1}, all germ cell types present, but slight reduction in round and elongating spermatids. (**d**) *Dazl^{Tm1Hgu}*/+ *Y^{d1}* (number 8), all germ cell types present, but a reduction in round and elongating spermatids. Tubular lumen larger than normal. (**e–j**) *Dazl^{Tm1Hgu}*/+ *Y^{d1}* (numbers 10 and 11). (**e**) Sertoli cell only tubule, with interstitial cell hyperplasia. (**f**) Pachytene arrest. (**g**) Apoptotic nuclei (arrows). (**h–j**) Symplasts or multinucleate aggregations (arrows). (**h** and **i**) Pachytene symplasts in tubules showing pachytene arrest. (**j**) Round spermatid symplast in tubule showing some round spermatid development. **a**–**g**: bar = 10 μm, **h**–**j**: bar = 40 μm.

Table II. Sperm count and testis weights for the different genotypes investigated

No.	Genotype	Testis weight (mg)	SEM	Sperm count per epididymis	SEM
1	+/+Y^{wt}	140	±13.28	86.70	±9.23
2	+/–Y^{wt}	72		35.00	
3	+/–Y^{wt}	95	±7.67	45.00	±5.33
4	+/–Y^{wt}	96		39.50	
5	+/+Y^{d1}	110		70.50	
6	+/+Y^{d1}	115	±7.67	75.00	±5.33
7	+/+Y^{d1}	112		66.50	
8	+/–Y^{d1}	75		32.00	
9	+/–Y^{d1}	74	±6.64	16.50	±4.62
10	+/–Y^{d1}	43		5.00	
11	+/–Y^{d1}	48		4.00	

The *Dazl*^{Tm1Hgu} allele is referred to as –, the wild type *Dazl1* allele as +; wt = wild type. SEM = standard error of means.

1980) and counter stained with 2% Giemsa (pH 6.8) for 5 min at RT. Slides were coded and scored blind to genotype. Various schemes for classifying sperm head morphology have been reported (Krzanowska, 1976; Mahadevaiah *et al.*, 1998). We most closely followed that of Mahadevaiah *et al.* (1998). Several additional minor categories observed in *Dazl*^{Tm1Hgu}/± and Y^{d1} mice have been included (Table I). Only complete spermatozoa with head and tail attached were scored to ensure consistency between scored numbers of different animals and to rule out preparation artefacts. Tail abnormalities were scored according to our own classification.

Testis sections

Sections 6 µm thick were prepared from testes fixed in Bouin's solution and then wax embedded. They were stained with haematoxylin and eosin by standard methods.

Statistical analysis

The results were analysed using Minitab12. General linear model (GLM) analysis of variance assumed a 2×2 cross-classification with the mean squares adjusted to allow for unequal replications of each genotype. Standard errors appropriate to each class shown in Tables I and II were calculated from the mean square errors in the analyses of variance and are employed in *t*-tests (with 7 degrees of freedom) of contrasts between the four classes. Homogeneity of variance tests (Bartlett's and Levine's) indicated that data transformation was only necessary for the 'grossly abnormal' percentages of sperm heads. These were analysed using a natural log transformation, hence standard errors are not shown in Table I.

Results

Sperm counts

Sperm counts and testis weights of the different genotypes were highly correlated (correlation coefficient of 0.969; Table II).

Although the *Dazl*^{Tm1Hgu}/+ males are fertile (Ruggiu *et al.*, 1997), the sperm count was considerably reduced, being only 45.9% ($n = 3$) of normal. Sperm counts for the Y^{d1} males were slightly impaired (Mahadevaiah *et al.*, 1998) having a mean count that was 81.5% of normal ($n = 3$). The combination of heterozygous *Dazl1* and Y^{d1} produced the most marked effect, the mean sperm count only being 16.6% of normal ($n = 4$). There was variation in this group, one animal resembled the *Dazl1* heterozygotes, two were extremely low at 5.2% of normal ($n = 2$), and one was intermediate between them.

The numbers of spermatozoa from the *Dazl*^{Tm1Hgu}/+ *Y*^{d1} mice were significantly less than both *Dazl*+/+ *Y*^{d1} and wild type Y ($P < 0.0001$) and also than the *Dazl*^{Tm1Hgu}/+ with wild type Y ($P < 0.01$). Statistical analysis of variance showed that the interaction between the effects of deletion and heterozygosity were negligible ($P = 0.485$). The depression in the sperm count for heterozygous Y-deleted males could be adequately explained by a main effect of heterozygosity added to a smaller main effect of Y deletion. In biological terms, this means that the effects of the two mutations are not synergistic.

Sperm morphology

Heads

Two features are important for assessing head morphology. Firstly the insertion of the mid-piece into the head under normal circumstances should have a clear stepped appearance (Table I). Secondly the overall shape of the sperm head must be considered. Table I shows two examples of each of the varying types of sperm head identified. It is apparent that within such subjective categories minor variations are common. The grossly abnormal sperm heads in categories 2–6, where both assessment factors are changed, present few problems in classification. The changes in categories 1–1d are more subtle, and can be described as:

1: Normal; 1a: Mid-piece insertion normal, head showing a flattened frontal curvature; 1b: Mid-piece insertion normal, head with a rear projection above the mid-piece; 1c: Mid-piece insertion normal, curved hook of spermatozoa malformed; 1d: Mid-piece insertion abnormal, head shortened with very angular outline, hook not fully formed.

The numbers observed in each category for each genotype are shown in Table I.

Categories 1c and 1d were observed predominantly in Y^{d1} and *Dazl*^{Tm1Hgu}/+ *Y*^{d1} males.

It has previously been shown (Bruce *et al.*, 1974) that sperm abnormalities can vary between 1 and 15% depending on strain. The figure of 2.5% for our control was at the lower limit. Analysis of variance showed very strong evidence ($P = 0.00002$) that there were fewer normal heads in the Y^{d1} groups than in the wild type Y-carrying groups (Table I). This was also the case for heterozygous *Dazl1* males compared to males with two wild type *Dazl1* alleles ($P = 0.001$). However, there is also considerable non-additivity since the two wild type Y-carrying groups do not differ significantly where there is strong evidence ($P = 0.00004$) that *Dazl*^{Tm1Hgu}/+ *Y*^{d1} has far fewer normal heads than +/+ *Y*^{d1} animals. The *Dazl*^{Tm1Hgu}/+ males exhibit a mean total of 11.2% abnormalities, Y^{d1} males 30.8% and *Dazl*^{Tm1Hgu}/+ *Y*^{d1} males 73.8%. The increased level in Y^{d1} over *Dazl*^{Tm1Hgu}/+ is due to the proportion of slight head abnormalities, the class 1a and 1b being far more frequent in the former. The elevated levels in the *Dazl*^{Tm1Hgu}/+ *Y*^{d1} males reflect increased numbers of all classes of grossly abnormal spermatozoa, particularly the class 4 (tulip-headed spermatozoa).

Table III. Numbers counted of normal and abnormal sperm tails

No.	Genotype	Tails						*n*
		normal		abnormal				
			%	a	b	c	%	
1	+/+Y^{wt}	190	95 ± 4.9	10			5 ± 4.9	200
2	+/–Y^{wt}	103		95	2			200
3	+/–Y^{wt}	111	57.83 ± 2.8	88	1		42.17 ± 2.8	200
4	+/–Y^{wt}	133		66	1			200
5	+/+Y^{d1}	175		24	1			200
6	+/+Y^{d1}	178	89.67 ± 2.8	22			10.33 ± 2.8	200
7	+/+Y^{d1}	185		15				200
8	+/–Y^{d1}	125		66	6	3		200
9	+/–Y^{d1}	138	65.25 ± 2.5	58	3	1	34.75 ± 2.5	200
10	+/–Y^{d1}	126		72	2			200
11	+/–Y^{d1}	133		65	2			200

Abnormal tails are categorized as a: bent and coiled tails; b: multiple tails; and c: multiple mid-pieces. n = total number of sperm tails counted. The *Dazl*^{Tm1Hgu} allele is referred to as –, the wild type *Dazl1* allele as +; wt = wild type.

Tails

Sperm tails can be divided into two regions, the mid-piece and the flagellum. Primary defects in each region such as multiple tails and bent or coiled tails may be due to defects in the structure of organelles such as the internal fibres or the outer sheath, and such spermatozoa have generally been considered incapable of fertilization. Multiple tails or mid-pieces (Figure 1a–c) were not seen in the control, they occurred at low levels in both the *Dazl*^{Tm1Hgu}/+ (0.7%) and Y^{d1} (0.2%) males, and while still at low levels were most frequent in the *Dazl*^{Tm1Hgu}/+ *Y*^{d1} males (2.1%) (Table III). Bent and coiled tails (Figure 1d–f) were most frequent in the *Dazl*^{Tm1Hgu}/+ (41.1%, n = 3). They were also present in the Y^{d1} males but about four-fold lower in frequency (10.1%, n = 3). Unlike sperm head abnormalities the frequency of tail defects in the *Dazl*^{Tm1Hgu}/+ *Y*^{d1} was not significantly increased in comparison with *Dazl*^{Tm1Hgu}/+ males (31.3%, n = 4) (Table III). However in these males the defects were qualitatively more severe with extreme coiling and an apparent self-adhesion of the mid-piece and flagella (Figure 1g–l). Minor structural defects were also seen in areas of both the mid-piece and flagella (Figure 1j–l), these often splitting into two sub-components.

Sections

The control male exhibited normal testis histology (Figure 2a). Tubules were uniform in cross sectional diameter and interstitial tissue was normal in appearance. All the stages of spermatogenesis were present. The Y^{d1} males appeared virtually normal (Figure 2c) with the occasional tubule containing a few apoptotic nuclei (1:100). The *Dazl*^{Tm1Hgu}/+ sections were similar to the Y^{d1}, except that in tubules at stage VII, elongating spermatids were reduced in number (Figure 2b). The *Dazl*^{Tm1Hgu}/+ *Y*^{d1} sections were variable in appearance. Sections from male number 8, with highest sperm count, appeared almost like *a Dazl*^{Tm1Hgu}/+. Tubule size was fairly uniform, while there was a marginal increase in interstitial tissue. All stages of spermatogenesis were present although numbers of elongating spermatids were reduced (Figure 2d). Multinucleate aggregations (symplasts) were seen in a few tubules (3:100) (Figure 2h–j). These have been reported in a variety of situations (MacGregor *et al.*, 1990; Odorisio *et al.*, 1998) where knock-out or transgenic technologies have disrupted normal spermatogenesis. They may be related to a destabilization of the cytoskeletal apparatus maintaining the integrity of intercellular bridges between the developing germ cells (Russell *et al.*, 1990). They usually originate from round spermatids, but other spermatogenic nuclei have been implicated (Ross *et al.*, 1998). Males 10 and 11, with the lowest sperm counts, showed the most severely impaired spermatogenesis. Tubular size was variable and interstitial tissue increased in both cases. Smaller tubules were occasionally Sertoli cell only (Figure 2e), while others showed pachytene arrest (Figure 2f). Round and elongating spermatids were severely reduced in larger tubules. The lumen of such tubules was also bigger than those of the other genotypes being filled with Sertoli cell cytoplasm. Tubules containing apoptotic nuclei (Figure 2g) were more frequent (12:100 and 26:100), as was the presence of symplasts (Figure 2h–j) in these males (26:100 and 32:100). The remaining male, number 9, was intermediate between these extremes, showing impaired spermatogenesis.

Discussion

This study was aimed (i) to investigate the resulting phenotype of combined genetic modifications of *Dazl1* and *Rbm* gene number in order to provide more evidence justifying a classification of both of them as candidates for an AZF, and (ii) to compare the phenotype with those of infertile or subfertile human individuals.

We have shown that the *Dazl1* heterozygote phenotype is a reduction of sperm output and tail abnormalities mainly affecting the mid-piece. The replacement of a normal Y with the Y^{d1} chromosome, accompanied by the induction of sex reversal through an *Sry* transgene, leads to an increased production of spermatozoa with abnormal heads. Although our numbers for Y^{d1} related head abnormalities differed slightly

from the figures reported by Mahadevaiah *et al.* (1998), the overall picture is the same. Mahadevaiah and colleagues' report is based on spermatozoa entering the epididymis. In contrast, we isolated spermatozoa from the entire epididymis. These different sources of spermatozoa might account for the differing values of abnormal spermatozoa obtained rather than age dependent variations.

Apart from the assigned predominant phenotypes we observed a small overlap in the phenotypes of the mutations, i.e. the number of abnormal sperm heads was slightly increased in *Dazl*^{Tm1Hgu}/+ compared to wild type animals, and a small proportion of spermatozoa in Y^{d1}-carrying animals exhibited tail defects. Y^{d1} animals have also a lower sperm count than wild type animals.

These slightly overlapping phenotypes were enhanced when the Y^{d1} chromosome was crossed onto a *Dazl*^{Tm1Hgu}/+ background resulting in a significant drop of the sperm count, an increase of abnormal sperm heads and more prominent midpiece defects of the tails. These data suggest a model in which *Rbm* genes (or in the case of the Y^{d1} chromosome all genes in the deletion), and *Dazl1*, affect at least three different phenotypic pathways in mouse spermatogenesis. It is difficult to assess whether both genes act in the same or in different molecular pathways. However, since all observed phenotypes seem to be more severe in *Dazl*^{Tm1Hgu}/+ *Y*^{d1} animals, this suggests an additive or synergistic effect, and we assume that *Rbm* and *Dazl1* are mainly acting in different molecular pathways. Since the phenotypes of both mutations on their own overlap only slightly, we conclude that the contribution of each gene to the main pathway of the other is only small.

Sperm head abnormalities and more strikingly sperm output vary between individual animals in *Dazl*^{Tm1Hgu}/+ *Y*^{d1} mice, whereas little variation is seen in all other genotypes studied here. It has been reported that Y chromosomes from different genetic backgrounds can affect the total percentage of sperm head abnormalities in inbred mouse strains (Krzanowska, 1976). An increase in specific types of abnormality has not been observed in the study of Krzanowska, in contrast to the situation observed in *Dazl*^{Tm1Hgu}/+ *Y*^{d1} animals. In these mice, the increase of specific abnormalities, e.g. the tulip-shaped class 4, is therefore not an effect of different genetic backgrounds, but is caused by reduced activity of the affected Y chromosomal genes and *Dazl1*.

A variant penetration of the phenotype in *Dazl*^{Tm1Hgu}/+ *Y*^{d1} was also observed in testis sections. Some animals, i.e. number 8, were only slightly affected by the reduction of functional *Dazl1* and *Rbm* genes and exhibited a mild phenotype that resembled a *Dazl*^{Tm1Hgu}/+ phenotype. The tubules of these animals had germ cells of all stages and a complete depletion of germ cells was never observed. In contrast, very badly affected animals (numbers 10 and 11) showed only few tubules with complete spermatogenesis but predominantly tubules with arrested pachytene stages or Sertoli cells only. Fertilization ability of these most severely affected animals has not been investigated.

The variation in severity of the double mutant phenotype could also be caused by strain-dependent segregation of different modifier genes. Although initially generated by modifying a C129-derived allele, the *Dazl1* null allele was maintained on a random bred MF1 strain. Crossing of this null allele in different inbred strains did not result in a variable phenotype (data not shown). Thus the *Dazl1* null phenotype seems not to be susceptible to the action of modifying genes. Variations of sperm output in different animals has not been observed for the Y^{d1} chromosome, which was also crossed onto the MF1 background (Mahadevaiah *et al.*, 1998). However, involvement of modifying genes cannot be ruled out to contribute to the observed individual variations in *Dazl*^{Tm1Hgu}/+ *Y*^{d1} males.

The variable penetration of the AZF phenotype is also apparent in human patients with deletions in the AZFc, ranging from Sertoli cell only to meiotic or spermatid maturation arrests that lead to absence of mature (azoospermia) or only few spermatozoa (oligozoospermia). In some cases increased rates of dysmorphic sperm heads (oligoasthenoteratozoospermia) have also been observed (Vogt *et al.*, 1996).

This variability might point to an involvement of other factors apart from the single candidate gene *DAZ*, influencing the severity of the AZFc phenotype in humans. Those factors can be environmental, epigenetic or genetic in nature.

The data presented in this study, however, show that the variable AZFc phenotype in humans is mimicked in the mouse by copy number reduction of *Dazl1* and *Rbm,* and/or additional Y chromosomal genes. Assuming similar functions during spermatogenesis for *Rbm* and *Dazl1* in humans and mouse, this observation provokes the question as to whether RBM is also involved in the AZFc phenotype. According to the data presented by Elliott and colleagues (1997) RBM expression from the minimal critical region in AZFb is unaffected in the AZFc patients studied, excluding a direct involvement of RBM itself. Another possible explanation would be the involvement of other genes that are localized in the vicinity of or in the murine *Rbm* cluster itself. Hence, the similar phenotype observed in this study would not reflect an effect mediated by the copy number reduction of *Rbm* but by the removal or silencing of those different genes, which might on the other hand be localized in the AZFc region on the human Y. Deletions in AZFc also reduce the distance between the heterochromatic region of the Y chromosome and active genes. Hence, position effects evoking silencing of active genes to various extents might contribute to the variability of the AZFc phenotype in some individuals.

In summary, this study has shown firstly that *Dazl1* and *Rbm* and/or other genes localized in the Y^{d1} deletion interval have multiple targets that contribute to different areas of the establishment and progression of spermatogenesis. Secondly, that a reduction of active gene product of those genes gives rise to a phenotype similar to the heterogeneous AZFc phenotype observed in humans.

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