Mini-Review

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Progress in understanding the molecular functions of DDX3Y (DBY) in male germ cell development and maintenance

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Summary **Human *DDX3* paralogs are housed on the X chromosome (*DDX3X*) as well as in the non-recombining region Yq11 of the Y-chromosome (*DDX3Y* or *DBY*). A gene encoding RNA helicase DDX3Y is located in the *AZoospermia Factor a* (*AZFa*) region of the Y-chromosome and expressed only in male germ cells. Deletions encompassing the *DDX3Y* gene lead to azoospermia and cause Sertoli Cell-Only Syndrome (SCOS) in humans. SCOS is characterized by a complete germ cell lack with preservation of somatic Sertoli cells. This review summarizes current advances in the study of DDX3Y functions in maintenance and development of early male germ cells. Data obtained from a mouse xenotransplantation model reveals that DDX3Y expression is enough to drive germ cell differentiation of *AZFa*-deleted human induced pluripotent stem cells (iPSCs) and for activation of the specific set of germline developmental genes. Results achieved using the testes of *Drosophila* demonstrate that DDX3Y homolog Belle is required cell-autonomously for mitotic progression and survival of germline stem cells and spermatogonia as the upstream regulator of mitotic cyclin expression.**

Keywords: Sertoli cell-only syndrome, DDX3Y, spermatogenesis, germline stem cells, cyclin B

1. Introduction

The most common cases of human male infertility which are caused by a failure of sperm production are associated with microdeletions in the *AZoospermia Factor* (*AZF*) region in the long arm of the Y chromosome (*1-4*). This region contains three loci that are termed *AZFa*, *AZFb*, and *AZFc* with corresponding deletions linked to male infertility (*3,5*) (Figure 1). It has been shown that the majority of the Y chromosome deletions originates *de novo* and putatively occurs in the father's germline (*6*). Since only a limited part of the Y chromosome pairs with the X chromosome and the *AZF* is located in the non-recombining region of the Y chromosome, the mechanism of emergence of the Y deletions is still unclear. It has been proposed that the Y deletions are the consequence of illegitimate intra-chromosomal meiotic recombination between highly repetitive sequences adjacent to the *AZF* loci (7,8).

The specific infertility phenotypes are associated with deletions in each locus (*3,9*). The *AZFa* deletions cause a complete absence of germ cells in the testis seminiferous tubules with preservation of somatic Sertoli cells (the so-called Sertoli Cell-Only Syndrome; SCOS) (*10,11*). This is the most severe azoospermia phenotype of the three deletion loci. The *AZFb* deletions lead to spermatocyte maturation arrest as well as the azoospermia phenotype. The *AZFc* deletions allow limited sperm production and thus are linked with hypospermatogenesis. The *AZFa* contains only two expressed genes both with X homologues that escape inactivation: *ubiquitin specific peptidase 9*, *Y-linked* (*USP9Y*) and *DEAD-box helicase 3*, *Y-linked* (*DDX3Y* or *DBY*). Both genes were initially considered as candidates for the male fertility factor. *USP9Y* encodes an ubiquitin-specific protease that promotes intracellular

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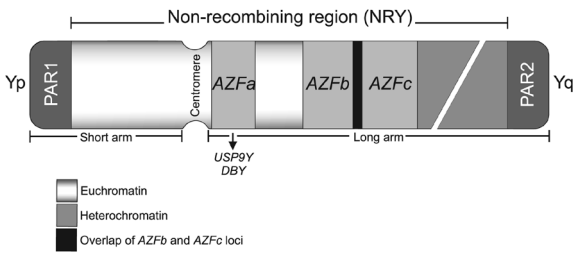

Figure 1. Representation of the *AZF* loci on the Y chromosome. PAR1 and PAR2 (shown in dark grey regions at the ends of the chromosome) are pseudoautosomal regions 1 and 2 of the Y chromosome. Outside the PARs *AZF* (*AZoospermia Factor*) loci are depicted in light grey and located in the non-recombining region (NRY). Yp and Yq designate the short and long chromosome arms of the chromosome, respectively. Adapted from (*2-4,15*) with modifications.

cleavage of ubiquitin molecules from ubiquitinated proteins (*3,9*). However, it was shown that USP9Y did not provide essential functions during spermatogenesis; mutations in *USP9Y* including deletion mutations were found to be transmittable in fertile patients (*12-14*). A second gene in the *AZFa*, *DDX3Y*, appears to represent the major spermatogenesis gene of this region (*15,16*). DDX3Y encodes an RNA helicase of the large DDX3 subfamily of the DEAD-box family of proteins, which are found from yeasts to humans. DDX3 helicases generally function in ATP-dependent RNA unwinding, strand annealing and remodeling of ribonucleoprotein complexes. They take part in most aspects of cellular RNA metabolism: mRNA nuclear export, transcriptional regulation, translation initiation, and also in cell cycle control and programmed cell death (*17-19*). The product of X-chromosomal homologue, DDX3X, is found ubiquitously in different human tissues, whereas translation of DDX3Y is restricted to male germ cells (*20*). DDX3X and DDX3Y share 91.7% homology in their amino acid sequence. Both proteins are expressed in the testes; however, DDX3Y is found predominantly at premeiotic stages in spermatogonial cells, whereas DDX3X is detected later in spermatids (*15,16,20*). This divergent expression pattern arises due to complex transcriptional and translational control mechanisms (*21,22*) and it seems to predetermine presumed specific functions of DDX3Y in premeiotic spermatogenesis steps. Testis-specific *DDX3Y* transcription initiates from a distal promoter that takes place within upstream Y-specific minisatellite *MSY2* repeats (*21*). This distal promoter region for *DDX3Y* on the Y chromosome appears to be conserved in primates; however, it is not active upstream of the mouse *Ddx3y* gene (*21*).

It has been shown that mouse homologue Ddx3y is expressed widely in all analyzed tissues (*23*). In contrast to humans mouse Ddx3y protein is found to be dispensable for spermatogenesis (*24*), but *Ddx3y* mRNA is retained in mouse spermatozoa and appears to contribute to early zygotic development (*25*). The mouse *Ddx3y* gene also has an X-chromosomal homologue *Ddx3x* (*26*). It is supposed that molecular functions of Ddx3y protein may be carried out by Ddx3x representing the same expression pattern in mouse testes (*23*). The third mouse DDX3 homologue PL10 (D1Pas1) is expressed only in the testes (*23,27,28*). The *PL10* gene is located on chromosome 1 and its expression is detected in pachytene spermatocytes and postmeiotic haploid spermatids (*23*). Thus, in spite of some similarities, the functional significance of DDX3 genes in spermatogenesis of humans and mice is quite different.

Progress in understanding the molecular functions of DDX3Y (DBY) in human male germ cell maintenance is challenging, because DDX3Y expression appears to be required for early stages of testis development in human fetal germ cells (prospermatogonia). Expression of DDX3Y during the second and third trimester of fetal testis development suggests its role in early germ cell differentiation and amplification and indicates that in individuals with a failure in DDX3Y expression germ cell loss may occur prenatally or during pre-pubertal development (*16,20*). Thus, specific functions of DDX3Y in germline development and maintenance are currently underinvestigated. In this review we focus on recently published studies that expand our understanding of the functions of DDX3Y as an essential spermatogenesis regulator.

2. DDX3Y genetically complements the *AZFa* deletion and restores germ cell development and transcription program

Mammalian spermatogenesis occurs in the seminiferous tubules of the testes. The seminiferous tubules are surrounded by the basement membrane, which isolates the tubules from the interstitial space containing myoid cells, Leydig cells and blood vessels (Figure 2). The seminiferous epithelium is composed of somatic Sertoli cells and developing germ cells at different stages of differentiation. Large undivided Sertoli cells are attached to the basement membrane, but they spread their cytoplasmic extensions towards the lumen of the tubules and surround multiple germ cells at all stages of differentiation. Spermatogonial stem cells (SSCs) originate from gonocytes (prospermatogonia), which in turn arise from primordial germ cells (PGCs) during fetal development. In the postnatal testes, SSCs are located on the basement membrane of the seminiferous tubules (Figure 2). SSCs undergo self-renewing and differentiating divisions to maintain continuous sperm production throughout male adulthood. In humans, the SSC pool is composed of A_{dark} and A_{pale} type spermatogonia. A_{dark} cells are reserved SSCs and they do not usually enter active mitotic divisions. On the contrary, A_{pale} spermatogonia are mitotically active and undergo one-two transit amplifying divisions generating type B spermatogonia. Type B spermatogonia give rise

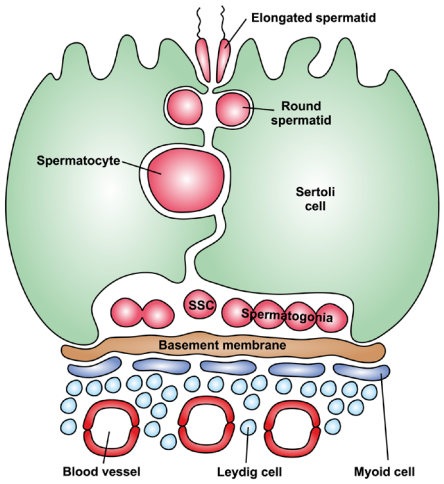

Figure 2. Scheme of human spermatogenesis. Simplified scheme of human spermatogenesis was adapted from (*30,31*) with modifications. The seminiferous tubules of the testes are surrounded by the basement membrane, which isolates the tubules from the interstitial space containing myoid cells, Leydig cells and blood vessels. The seminiferous epithelium is composed of somatic Sertoli cells and germ cells at different stages of differentiation. Spermatogonial stem cells (SSCs) are located on the basement membrane. Sertoli cells are also attached to the basement membrane, but they extend towards the lumen of the tubules and contact with germ cells at all stages of differentiation. SSCs undergo self-renewing and differentiating divisions to provide germ cells of the subsequent stage, type B spermatogonia. Type B spermatogonia give rise to primary spermatocytes. Then primary spermatocytes enter the first meiotic division to yield secondary spermatocytes, which divide into haploid spermatids migrating towards the lumen. Finally, mature spermatozoa are released into the lumen of the seminiferous tubules.

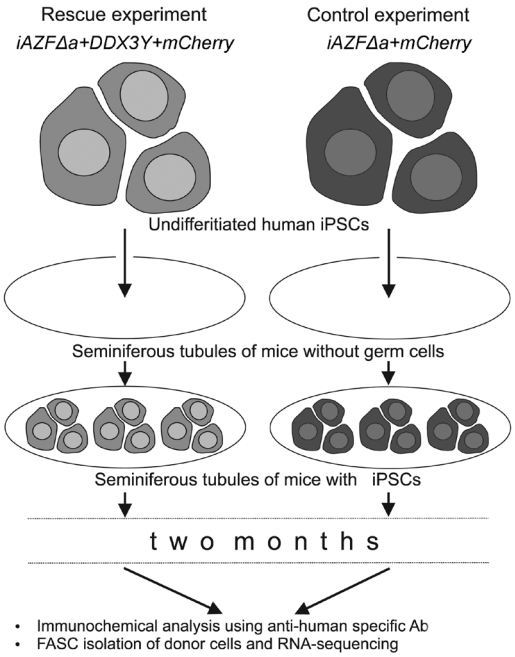

Figure 3. General scheme of the xenotransplantation assay of Ramathal *et al*. (*32*). Cell suspensions of human iPSCs with the *AZFa* deletion (*AZFΔa*) carrying corresponding transgene constructs are transplanted into the testis of busulfan-treated infertile nude mice. Two months following the transplantation, the testes are recovered and the seminiferous tubules are dissected for subsequent analysis.

to primary spermatocytes. Primary spermatocytes enter the first meiotic division and divide into secondary spermatocytes, which in turn divide into haploid spermatids, which migrate towards the lumen where terminally differentiated spermatozoa are released into the lumen (*29-31*).

To examine the functions of the *AZFa* gene *DDX3Y* in germ cell development and maintenance Ramathal and coworkers (*32*) used a mouse xenotransplantation model established previously. Mouse seminiferous tubules proved to be potent for induction of germ cell formation from human induced pluripotent stem cells (iPSCs) (*30,33*). It was shown that undifferentiated human iPSCs after being transplanted into mouse seminiferous tubules undergo differentiation to germ-cell-like cells (GCLCs) located close to the basement membrane and expressing cell-specific markers of PGCs.

Ramathal and coworkers used the iPSC line with the *AZFa* deletion (*AZFΔa*) that was genetically complemented with the *DDX3Y* gene (*32*). For this study transgene constructs carrying either FLAG-tagged *DDX3Y* with the *mCherry* marker gene (rescue lines) or *mCherry* marker alone (mutant control lines) were integrated in human iPSCs that harbored *AZFΔa*. The authors transplanted the undifferentiated iPSCs from the rescue and control lines into mouse seminiferous tubules of immunodeficient mice with endogenous spermatogenesis drug-eliminated in advance (Figure 3). After two months the post-transplantation xenografts were analyzed by immunohistochemistry to reveal human germ-cell like cells (GCLCs) using marker antibodies against NuMA (human cell-specific protein), Vasa (basic germline marker) and DDX3Y. Whereas the *AZFΔa* mutant line did not have cells that were positive for DDX3Y, all NuMA-positive GCLCs from the rescue lines were DDX3Y-positive and the majority of DDX3Y-positive GCLCs were also VASA-positive. The tubules, which were transplanted with the rescue lines, contained significantly more VASA-positive GCLCs in comparison with the tubules with *AZFΔa* mutant line xenografts. Human spermatogonia-specific marker proteins DAZ1 and UTF1 were not expressed in mouse seminiferous tubules transplanted with *AZFΔa* mutant iPSCs. However, these proteins were found in a subset of GCLCs from the *DDX3Y*-rescued lines. Thus, the authors have detected a quantitative enhancement in GCLC formation as well as germ cell differentiation in all the rescue lines.

The authors succeeded in purifying the germ cell fractions from mouse xenografts and subsequent sorting of donor cells positive for mCherry. Analysis of the transcriptome by RNA-Seq reveals that the transcription profile of DDX3Y-rescued GCLCs is significantly different from the *AZFΔa* mutant GCLCs, which exhibit a profile closely resembling undifferentiated

donor iPSCs. 248 transcripts were found to be differentially expressed between undifferentiated iPSCs, mutant GCLCs and rescue GCLCs. A large subset of transcripts which were up-regulated in iPSCs and mutant GCLCs were absent or significantly down-regulated in *DDX3Y*-rescued GCLCs. Note that the upregulated set of non-complemented *AZFΔa* GCLCs was found to be strongly enriched in pluripotency genes (including *POU5F1*, *LIN28A*, *SOX2 NANOG* and *MYC*) in contrast to *DDX3Y*-complemented GCLCs. A group of genes that specifically are up-regulated in the rescue GCLCs includes zinc-finger transcription factors, RNA-metabolism modulators, cell cycle and cell-cell communication genes. Thus, *DDX3Y*-complemented GCLCs in the mouse xenograft system are able to differentiate to the prospermatogonial stage and maintain a specific transcriptional program that is similar to that of PGCs and prospermatogonia.

3. *Drosophila homologue* of DDX3Y RNA-helicase Belle provides essential cell-autonomous functions in the male germline

In another recently published paper the testes of *Drosophila* were used as a relatively simplified model to study DDX3 functions in spermatogenesis (*34*). For *Drosophila*, effective approaches have been developed to turn on or off the expression of a selected gene in specific tissues or cells. Among them are mosaic clonal analysis (*35*); inducible RNAi-mediated gene knockdowns that could be ectopically induced in certain cells using *GAL4-UAS* system and tissue-specific promoters (*36,37*); and the CRISPR RNA/Cas9 system allowing precise gene engineering (*38*). All of them can be successfully applied for the study of spermatogenesis. Another great advantage of *Drosophila* testes as a model system consists in the possibility of studying germline stem cells and other testis cells *in situ*, using immunofluorescence confocal microscopy (*39*).

The apical testis tip of an adult fly is occupied by a somatic niche structure called the hub (Figure 4), which supports two adjacent stem cell populations: germline stem cells (GSCs) and somatic cyst stem cells (CySCs) (*40-42*). The self-renewing division of a GSC produces a new GSC and a goniablast (spermatogonium). The division of a CySC generates a self-renewing CySC and a cyst cell. A pair of somatic cyst cells encapsulates spermatogonium thus forming a functional unit of spermatogenesis – the cyst. Cyst cells are considered as mammalian Sertoli cell analogs, despite evident structural differences between the organization of the testes in *Drosophila* and humans (*41*). Spermatogonial cells within cysts undergo four rounds of mitotic amplification divisions resulting in 16-cell cysts of interconnected primary spermatocytes, each surrounded by two undivided cyst cells. Primary spermatocytes in the cyst undergo a long growth phase. Then they enter

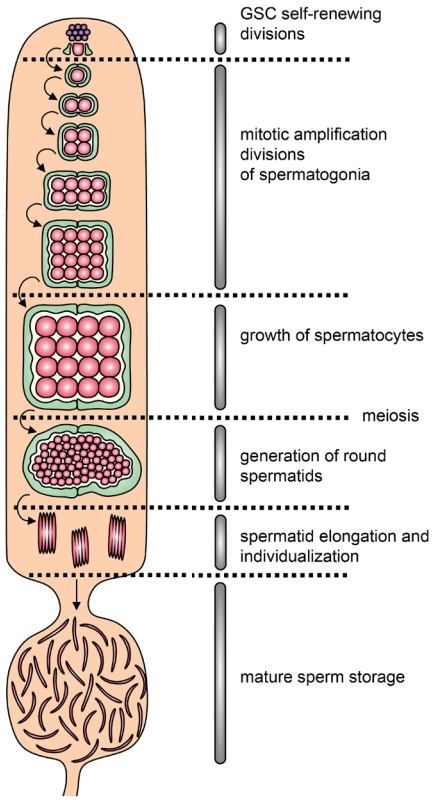

Figure 4. Representation of *Drosophila* spermatogenesis stages. At the apical testis tip germline stem cells (GSCs, red) are located adjacent to the hub (violet) and are surrounded by two somatic cyst stem cells (CySCs, green) (top of the figure). One of the daughter cells of GSC, the goniablast (red), undergoes four cell divisions to create cysts of 16 spermatogonia. Then spermatogonia switch to the spermatocyte developmental program. Mature spermatocytes synchronously enter meiosis producing 64 haploid spermatids. Finally, elongated spermatids undergo individualization at the basal end of the testis and enter the seminal vesicle, where mature sperm is stored until copulation (bottom part of the figure).

a synchronous meiotic division producing 64 haploid spermatids (Figure 4).

Drosophila RNA-helicase Belle is a single homolog of human DDX3 proteins due to the non-redundancy of protein-coding genes in the fly genome. All known *belle* null mutant alleles are lethal, suggesting that Belle has essential functions for fly viability. It was also shown that Belle was genetically required for male and female fertility; however, its molecular functions in gametogenesis had been obscure (*43*). Kotov and coworkers used flies with viable heteroallelic combinations of *belle* mutations as well as germline-specific *belle-RNAi* knockdown flies (*34*). Using immunofluorescence staining and confocal microscopy of testis preparations they found that a deficiency of *belle* led to drastic germ cell depletion in the testes of newly eclosed males, while somatic cyst cell and hub cell populations were maintained. The observed developmental disorder recapitulates the SCOS phenotype in humans carrying the *AZFa* deletions and also leads to severe azoospermia. No

GSCs were detected in the testes of *belle*$^{6/neo30}$ mutants or males with a germline *belle-RNAi* knockdown in comparison with the wild-type control (0 cells versus 9.3-9.6 cells per testis of control males). Using TUNEL assay, Kotov and coworkers found that germ cells in the *belle*$^{6/neo30}$ testes as well as in the case of a germline-specific *belle-RNAi* knockdown underwent premature cell death through apoptosis. The authors conclude that Belle is required cell-autonomously for the maintenance of early germ cells including GSCs, because *belle-RNAi* knockdown in testis somatic cells does not result in the loss of GSCs and germ cells of subsequent stages.

Kotov and coworkers also showed that the testes of *belle*$^{6/neo30}$ third instar larvae still contained all populations of early germ cells in contrast to the testes of adult flies (*34*). This observation made it possible to propose that PGCs correctly migrated into embryonic gonads during mid-to-late embryogenesis in *belle* mutants. However, while analyzing *belle* larval testes

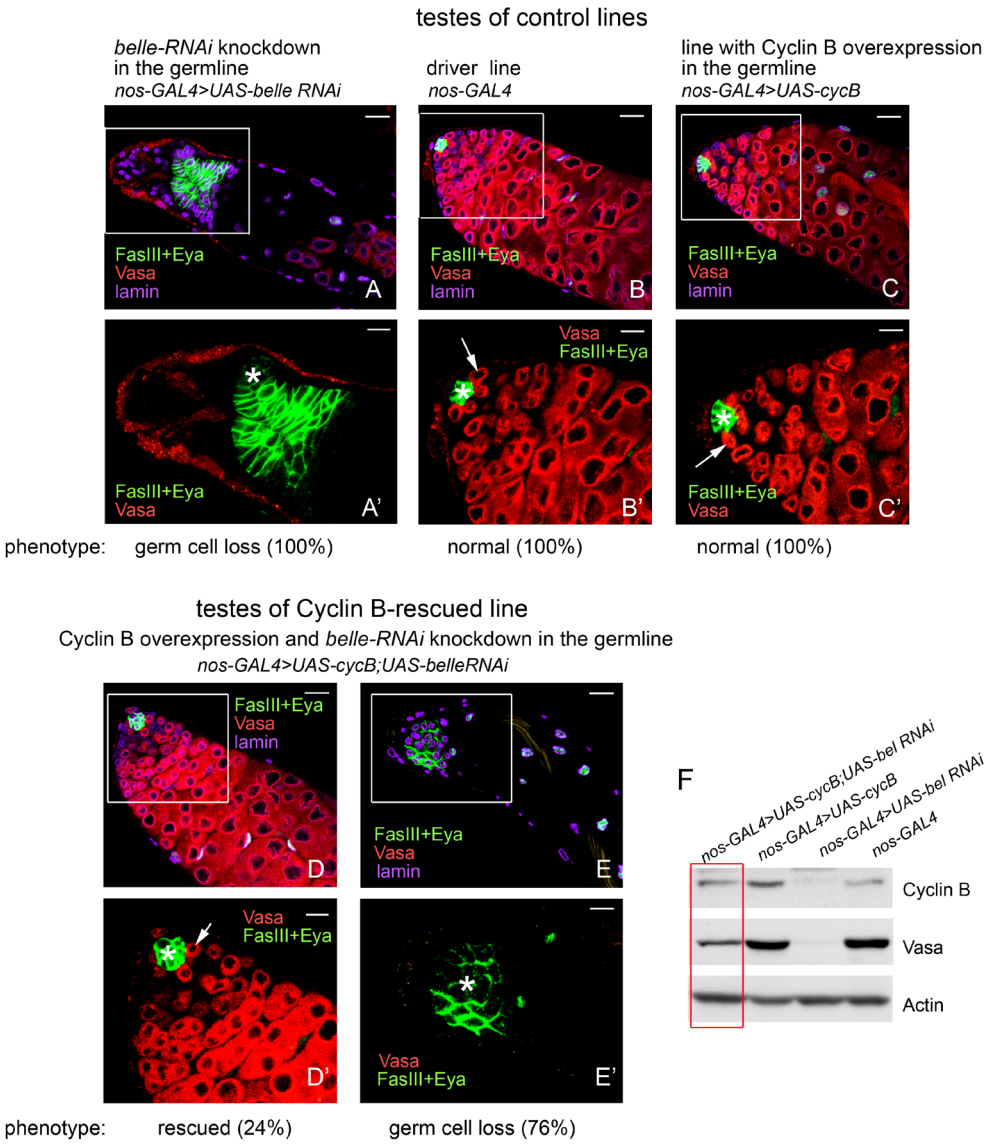

Figure 5. Transgenic overexpression of Cyclin B partially rescues spermatogenesis in the *belle-RNAi* knockdown testes. Immunofluorescent analysis of the testes of control lines **(A-C)** and Cyclin B-rescued line **(D, E)** obtained in rescue experiments. **(A-E)** Testes were stained with anti-Fas III (green, marker of the hub), anti-Eya (green, marker of mature cyst cells), anti-Vasa (red, germline marker) and anti-lamin (violet, marker of the nuclear envelope) antibodies. Scale bars are 20 μm. Bottom panels **(A'-E')** show higher magnifications of the apical tips of the testes shown in the white boxes of the corresponding top panels (without lamin staining). Where applicable, white arrows indicate one of the GSCs identified as a Vasa-stained germ cell attached to the hub (asterisks). Scale bars are 10 μm. **(D, D')** A portion of *nos-GAL4>UAS-cycB;UAS-belle RNAi* flies (24%) restores a wild-type phenotype of the testes with proper maintenance of GSCs. **(E, E')** The other portion of *nos-GAL4>UAS-cycB;UAS-belle RNAi* males has testes with reduced size that contain almost no germ cells while preserving mature cyst cells. **(F)** Western blot analysis of testis lysates from fly lines of the rescue experiment. A half-restored Vasa (germ cell marker) protein level is found in the *nos-GAL4>UAS-cycB;UAS-belle RNAi* testes (red outline). Actin stained with anti-Actin antibodies is used as a loading control. The figure was reproduced from (*34*) with permission from Elsevier.

Table 1. Functions of mitotic cyclins in the cell cycle of *Drosophila* male GSCs

Cyclin	Corresponding phase of cell cycle	Functions	Defects in male GSCs maintenance owing to the disruption of cyclin expression	*Ref.*
Cyclin A	end of S – G2 – start of M	Cyclin A in complex with either Cdc1 or Cdc2 takes part in the continuation of S phase	centrosome misorientation	(*44*)
		Maintenance of centrosome orientation in the proximity of the hub-GSC junction (centrosome orientation checkpoint)		
Cyclin B	M	Cyclin B-Cdc2 complex is responsible for entry into mitosis	G2-M transition arrest	(*34,45,46*)
			Loss of GSCs and germ cells of subsequent stages through cell death	

morphology, the authors observed a partial germ cell loss along with the presence of abnormally large early germ cells with hallmarks of G2 mitotic delay. In agreement with these observations, the authors detected a considerable decline in expression of the major mitotic cyclins, A and B, in the testes of hypomorph $belle^{EY08943/neo30}$ mutants owing to reduction of their transcription levels. The authors suggested that early germ cells including GSCs in the testes of *belle* mutants could not enter mitosis owing to an insufficient dose of mitotic cyclins. A failure to enter mitosis appears to trigger programmed cell death and leads to the germ cell loss phenotype. In support of this hypothesis, Kotov and coworkers achieved a partial but significant rescue of germ cell survival, mitotic progression (Figure 5) and male fertility by germline-specific overexpression of Cyclin B in the *belle-RNAi* knockdown testes (*34*). Overexpression of Cyclin A did not rescue the *belle-RNAi* knockdown phenotype. These results support an important role of mitotic cyclins in development and survival of early germ cells, including GSCs. Current data about the functions of major mitotic cyclins in the male GSCs cell cycle and survival are summarized in Table 1. It has been previously shown that a mutational depletion of Cyclin B led to a total lack of germ cells in the testes, and transgene overexpression of Cyclin A did not restore the germ cell loss phenotype in *cyclin B* mutants (*46*). Thus, at least one crucial function of Belle in early germ cells is the maintenance of Cyclin B level for ensuring mitotic divisions.

Overexpression of Cyclin B in the germline led to an almost complete restoration of early germ cells in the testes of $belle^{EY08943/neo30}$ mutants, but in this case male fertility was not restored (Kotov, Olenkina unpublished data), indicating additional functions of Belle during the subsequent steps of spermatogenesis. Indeed, it has been previously shown that the testes of hypomorph *belle* mutants exhibited defects in meiotic chromosome segregation and cytokinesis with a high frequency, as well as a disorganization of elongated spermatid bundles (*43*). RNA helicase Belle in flies presumably combines the functions of both human DDX3 homologues. While DDX3Y is expressed at the premeiotic stage in spermatogonia, DDX3X expression is observed later in spermatids in the human male germline (*15,16,20*). Taking into account that the understanding of the functions of DDX3X at the late stages of human spermatogenesis is rudimentary, the employment of the *Drosophila* model may contribute to elucidation of DDX3 protein functions in the maintenance of spermatogenesis in humans.

4. Conclusions

The data presented by Ramathal and coworkers clearly demonstrate that DDX3Y expression is enough for driving germ cell differentiation of *AZFa*-deleted iPSCs and for induction of germline developmental gene expression (*32*). The obtained results suggest that DDX3Y functions are required during the early developmental stage in humans that include a period from PGC specification to prospermatogonial cell development. The expression of early spermatogonia-specific marker proteins indicates that DDX3Y-rescued GCLCs are able to progress to the prospermatogonial stage and that DDX3Y regulates this transition. Taken together, these results demonstrate for the first time that DDX3Y has essential functions in early male germline development in humans.

It has previously been assumed that DDX3Y protein is involved in the control of premeiotic proliferation of germ cells (*16*). To date, regulation of the cell cycle in human spermatogonia is poorly understood (*47*). A disruption of the *Drosophila* DDX3Y homologue Belle leads to severe germ cell loss through apoptosis in the testes, while somatic cyst cells are retained intact (*34*). Considering that cyst cells in *Drosophila* testes appear to be the functional analogs of Sertoli cells of mammalian testes, this phenotype mimics that of SCOS in humans with DDX3Y expression failure as a result of a corresponding deletion. Taking into account the conserved nature of DDX3 proteins in eukaryotes, Kotov and coworkers (*34*) demonstrate that Belle is required cell-autonomously for mitotic progression and survival of GSCs and spermatogonia as the upstream regulator of mitotic cyclin expression. These results obtained using

the *Drosophila* model support the importance of the tight regulation of mitotic cyclins in the fate determination of early germ cells. Both papers discussed here provide a significant insight into the functions of DDX3Y in spermatogenesis, suggesting that DDX3Y must be considered as en essential contributor to early male germ cell development and maintenance.

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