RESEARCH

AZFa candidate gene *UTY* and its X homologue *UTX* are expressed in human germ cells

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Abstract

The Ubiquitous Transcribed Y (*UTY* a.k.a. *KDM6C*) AZFa candidate gene on the human Y chromosome and its paralog on the X chromosome, *UTX* (a.k.a. *KDM6A*), encode a histone lysine demethylase removing chromatin H3K27 methylation marks at genes transcriptional start sites for activation. Both proteins harbour the conserved Jumonji C (JmjC) domain, functional in chromatin metabolism, and an extended N-terminal tetratricopeptide repeat (TPR) block involved in specific protein interactions. Specific antisera for human UTY and UTX proteins were developed to distinguish the expression of both proteins in human germ cells by immunohistochemical experiments on appropriate tissue sections. In the male germ line, UTY was expressed in the fraction of A spermatogonia located at the basal membrane, probably including spermatogonia stem cells. UTX expression was more spread in all spermatogonia and in early spermatids. In female germ line, UTX expression was found in the primordial germ cells of the ovary. UTY was also expressed during fetal male germ cell development, whereas UTX expression was visible only at distinct gestation weeks. Based on these results and the conserved neighboured location of *UTY* and *DDX3Y* in Yq11 found in mammals of distinct lineages, we conclude that *UTY,* such as *DDX3Y,* is part of the Azoospermia factor a (AZFa) locus functioning in human spermatogonia to support the balance of their proliferation-differentiation rate before meiosis. Comparable UTY and DDX3Y expression was also found in gonadoblastoma and dysgerminoma cells found in germ cell nests of the dysgenetic gonads of individuals with disorders of sexual development and a Y chromosome in karyotype (DSD-XY). This confirms that AZFa overlaps with GBY, the Gonadoblastoma susceptibility Y locus, and includes the *UTY* gene.

Lay Summary

AZFa Y genes are involved in human male germ cells development and support gonadoblastoma (germ cell tumour precursor cells) in the aberrant germ cells of the gonads of females with genetic disorders of sexual development. The AZFa *UTY* gene on the male Y chromosome is equivalent to *UTX* on the female X chromosome. These genes are involved in removing gene regulators to enable activation of other genes (i.e. removal of histone methylation known as epigenetic modifications). We wanted to learn the function of UTY and UTX in developing sperm and eggs in human tissues and developed specific antibodies to detect both proteins made by these genes. Both UTY and UTX proteins were detected in adult and fetal sperm precursor cells (spermatogonia). UTX was detected in egg precursor cells (primordial germ cells). UTY was detected in gonadoblastoma and dysgerminoma tumour cells (germ cell tumours originating from genetic disorders of sexual development due to having a Y chromosome). Based on our study, we conclude that UTY is not only part of AZFa, but also of GBY the overlapping gonadoblastoma susceptibility Y region.

Introduction

The human *UTY* (Ubiquitous Transcribed Y; a.k.a *KDM6C*) and *UTX* (Ubiquitous Transcribed X; a.k.a. *KDM6A*) genes (OMIM acc.: *UTY*: 400009; *UTX*: 300128) are functional members of the histone lysine demethylase 6 (*KDM6*) gene family (*UTY: KDM6C*; *UTX: KDM6A*) capable of removing histone 3 specific lysine (K) 27 methyl marks, H3K27me2/3, from chromatin at genes transcriptional start sites (TSS); thereby activating them for expression (Walport *et al.* 2014, Gazova *et al.* 2019). KDM6 proteins are characterised by the highly conserved Jumonji C (JmjC) domain functional in chromatin metabolism (Accari & Fisher 2015). Chromatin domains marked with H3K27me2/3 sites are repressed by binding of the methyltransferase Polycomb Repressor Complex 2 (PRC2). PRC2 binding is enriched in embryonic stem cells at promoters of genes encoding key developmental transcription factors and components of critical signalling pathways to promote cellular differentiation (Pasini *et al.* 2008). Methylation of H3K27 sites is therefore considered to be an epigenetic mark for maintaining embryonic stem (ES) cell pluripotency. Their plasticity is controlled by distinct H3K27me2/3 demethylases encoded by the *KDM6* genes, also expressed during early embryonic development (Welstead *et al.* 2012). Maintenance of dynamic steady-state levels of dimethylation and trimethylation of histone 3 lysine 27 (H3K27me2/3) in embryonic stem cells indicates thus a functional balance of expression of PRC2 and KDM6 proteins in these cells (Lee *et al.* 2007, Pasini *et al.* 2008).

Consequently, some developmental disorders are associated with dysfunction of this essential network control mechanism (Faundes *et al.* 2018). In human, microdeletions and point mutations of *UTX* cause Kabuki syndrome characterised by unique facial appearance, growth retardation, skeletal abnormalities and intellectual disability (Banka *et al.* 2015). Variable *UTY* and *UTX* expression profiles are found in distinct cancer cells suggesting some allelic compensation in the case of *UTY* for *UTX* deletions present in distinct groups of medulloblastoma (Robinson *et al.* 2012) as well as other human cancer cell lines (van Haaften *et al.* 2009).

UTY deletions seem not to be associated with somatic pathologies, although the genes transcripts and proteins were found to be expressed in multiple human tissues and leukocytes (Lahn & Page 1997, www.uniprot.org/uniprot/O14607). It has, therefore, been argued that UTY proteins are probably only required for some male cell-specific functions such as expressing a specific H-Y antigen (Warren *et al.* 2000) being restricted to some male-specific organs, such as the prostate (Dutta *et al.* 2016).

However, *UTY* deletions may cause complete absence of male germ cells because this Y gene is part of AZFa, a genomic Y region in the proximal part of the long arm of the human Y chromosome (Yq11.1) conserved in mammals since at least 70 Mya (Vogt *et al.* 2007). AZFa has a genomic extension of 1.1 Mb and includes a microdeletion found as *de novo* mutation event in about 9% of infertile men with a severe testicular pathology including complete germ cell aplasia (Kamp *et al.* 2001). AZFa is thus part of the Azoospermia Factor (AZF) locus mapped to distinct parts of the long Y arm (Tiepolo & Zuffardi 1976, Vogt *et al.* 1996). Recently it has been shown that *AZFa* might overlap with the *Gonadoblastoma Y* (GBY) locus in proximal Yq11, including the AZFa candidate genes, *USP9Y, DDX3Y,* and *UTY*, respectively (Vogt *et al.* 2019). Indeed, protein expression of the AZFa candidate gene *DDX3Y* in the germ cells of individuals with disorders of their sexual development (DSD) and a Y chromosome in their karyotype (DSD-XY individuals) seems to contribute to the susceptibility function of GBY to induce gonadoblastoma cells in these germ cells.

Since single *USP9Y* deletions can be inherited (Luddi *et al.* 2009). Therefore, single *DDX3Y* deletions are considered to cause mainly the severe testicular pathology of AZFa deletions (Tyler-Smith & Krausz 2009). This assumption gained support by the finding that DDX3Y proteins are only expressed in pre-meiotic spermatogonia (Ditton *et al.* 2004). However, nothing is yet known about the expression of UTY in the human male germ line and single *UTY* gene deletions in men have not yet been reported.

In the mouse, only *Jmjd3* (*Kdm6b*), of the *KDM6* gene family is functionally expressed in spermatogonia (Iwamori *et al.* 2013). Also, the *KDM6* antagonist *EZH1,* encoding the H3K27 methylation enzyme in the PRC2 complex is mainly expressed in mouse spermatogonia (Mu *et al.* 2017). It is therefore most likely, that also in human *KDM6* genes are functional in the germ line.

In this paper, we wanted to clarify this for the X-Y homologous, *UTY* and *UTX* gene pair asking the question (a) in which phase of male germ line UTY and UTX proteins are expressed predominantly; (b) whether UTX proteins are also expressed in female germ cells? Since there are no commercial UTY and UTX specific antisera available, that is, not cross reacting also to the homologous UTX, respectively, UTY proteins, we had to generate reliable UTY and UTX-specific antibodies as a first step.

Materials and methods

Human tissue sampling and analysis

All human testis and ovarian tissue sections analysed with distinct antibodies in our immune-histochemical experiments were collected only after written consent was obtained from the patients. Accordingly, the studies were formally approved by the local ethics committees of the University of Copenhagen for the fetal samples (4 specimens collected from gestation week (gw) 16, 25, 33, and 41, respectively, as indicated in the figure legends; and of the University of Heidelberg and of Bonn for the adult samples (4× KOHO3 specimens always used as the control sample for testis sections; 2× ovarian tissue sections from sample #1469/99; 4× DSD-46,XY: 2× gonadal tissue sections from GBY88 Swyer patient with Gonadoblastoma nests (see also Vogt *et al.* 2019) and from 2× gonadal tissue sections from GBY121 Swyer patient with dysgerminoma. Collection of more than one sample for each experiment from each of the specimen listed above was not possible due to the low number of tissue samples available in the clinic from the fetal germ lines, respectively, from the small aliquots of tissue samples got from each adult patient when suffering from gonadoblastoma or dysgerminoma, respectively. Pathologic assessment of gonadal histology was performed using current terminology (Ulbright & Young 2014).

Preparation of specific polyclonal UTY and UTX antisera

The human UTY and UTX proteins are strongly conserved along the complete protein-coding sequence (89%). Commercially available UTY antisera analysed were found to recognise also the UTX protein (data not published). Since our research questions definitely require specific UTY antibodies marking only UTY proteins in the testicular tissue section, we selected the 'VEKLLSSGAF' peptide of UTY (UTY protein sequence pos: 609–618 aa; accession no.: NP001245178.1; named UTY-1) and the 'NLPQGSLQNKTKLLPSIEF' peptide of the UTX protein (UTX protein sequence pos: 403–421 aa; accession no.: NP001278344; named UTX-1) as putatively specific antigen targets for preparation of specific UTY and UTX antisera in rabbits. They were synthesised by Peptide Speciality Laboratories GmbH (Heidelberg, Germany); conjugated to a carrier protein (maleimide-activated keyhole limpet haemocyanin; KLH; 1 mg peptide/1 mg KLH) via C-terminal cysteine residues using the Inject

Activated Immunogen Conjugation Kit (Pierce) and finally subcutaneously injected into New Zealand White rabbits following a standard booster protocol. Specificity and titre of the antisera were analysed by hybridisation to ovalbumin coupled parental peptides spotted at different concentrations onto nitrocellulose membrane stripes (Sartorius, Goettingen, Germany). The UTY-1 antiserum detected only the corresponding UTY-1 peptide; the UTX-1 antiserum only the corresponding UTX-1 peptide. Positively reacting antisera were enriched from the crude blood serum by ammonium sulphate precipitation and purified by affinity chromatography (SulfoLink Coupling Gel Support resin; Pierce). All fractions containing a positive cross-reaction to only their parental peptide were brought to neutral pH conditions with 1 M Tris buffer (pH 8) and stored in siliconised tubes with 50% glycerol at −80°C.

Preparation of UTY and UTX His-tagged recombinants expressed in M15 (pREP4) cells

His-tagged UTY- and UTX-recombinants expressed in M15 (pREP4) cells were constructed by cloning a cDNA sequence of the *UTX* exon 13–17 region including the UTX-1 target peptide in exon 13 and a cDNA sequence of the exon 16–18 region including the UTY-1 target peptide in exon 17 using the KpnI and SalI cloning sites of the pQE80L expression vector (The QIAexpressionist™; Qiagen).

Expression of the His-tagged-UTY- and UTX-recombinants in M15 (pREP4) cells was induced with 1 mM 1 mM IPTG (0, 30 and 60 min) and increasing quantities detected on western blots after incubation with Tetra-His antiserum (Qiagen). Two clones with the highest expression titre (UTX-1K37 and UTY-1 K36) were preserved with 15% glycerol at −80°C. Large quantities of proteins from the selected UTX-1 K37 and UTY-1 K36 recombinants were prepared in 200 ml LB medium after growing the cells to OD_{600} ~0.9–1.0 and 60 min induction with 1 mM 1 mM IPTG. The recombinant UTX and UTY proteins were purified after sonication (5 s bursts with microtip) by His-Tag affinity chromatography using an imidazol gradient between 0.1 and 0.25 M. Fractions of the pure protein were collected and stored at −80°C.

Western blots

For Western blotting experiments 2 μg of the purified recombinant UTX-1K37 and UTY-1K36- proteins were two times loaded in parallel onto a 15% SDS–PAGE for size fractionation; then, electrophoretically transferred onto

an Immobilon-P membrane (MilliporeEschborn). After pre-incubation of the membranes in blocking solution (5% skimmed milk powder in 1× TNT (50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.05% Tween 20; pH 7.6)) for 60 min at room temperature, the two UTX-1K37-UTY-1K36 blot doublets were separated and one stained for UTY with the affinity-purified UTY-1 antiserum, the second stained for UTX with the UTX-1 antiserum (1:150, v/v) overnight at 4°C. Visualisation of the hybridisation pattern was achieved by incubation with an anti-rabbit IgG-peroxidase conjugate (1:20,000, v/v) as secondary antibody (Dianova, Hamburg, Germany) in the same blocking solution for 60 min and using the Western Lightning Chemiluminescence Reagent Plus kit (Perkin Elmer, Langen, Germany) according to the manufacturer's instructions.

Immunohistochemical experiments with UTY and UTX specific antisera UTY-1, UTX-1

Immunohistochemical experiments with the polyclonal UTY and UTX specific antibodies for analysis of its expression in fetal (only UTY-1) and adult serial paraffin fixed human testis tissue section, respectively, in gonadal tissue sections of distinct DSD-XY individuals (only UTY-1) were performed as follows: for all experiments, we used 4–5 μm sections from tissue samples fixed in buffered formaldehyde, or in Bouin's fixative and subsequently embedded in paraffin. They were dewaxed, and then rehydrated in decreasing concentrations of ethanol. Immunohistochemistry staining was carried out with a standard indirect peroxidase method as described by Gueler *et al.* (2012). In short: antigen retrieval was achieved by incubation of the slides overnight with 0.2 M boric acid, pH 7 at 60°C. After washing in permeabilisation buffer (0.1 M Tris, 0.1 M NaCl, 0.1% Triton X-100; pH 7.4), endogenous peroxidase was quenched by incubation in 3% v/v hydrogenperoxide in methanol for 10 min at room temperature followed by 3% v/v goat serum (NGS) in permeabilisation buffer for 1 h to block unspecific Ig-binding sites. Sections were incubated overnight at 4°C with diluted primary polyclonal UTY-1, UTX-1, DBY-10 (for specific DDX3Y detection) antibodies using the following antisera dilutions: UTX-1 (1:60), UTY-1 (1:60). DBY-10 (1:500). A secondary biotinylated goat-anti-rabbit antibody was applied, followed by incubation with an avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA or Zymed, San Francisco, CA, USA). Finally, slides were stained with DAB (3,3'-diamino-benzidine tetrahydrochloride), counterstained with Mayer's haematoxylin and mounted in Immuno-Mount.

Results

UTY-1/UTX-1 antisera only mark their parental peptides on Western blots

The specificity of our polyclonal UTY-1 and UTX-1 antisera prepared for detection of only UTY and not UTX proteins, or of only UTX and not UTY proteins, respectively, was controlled by using specific peptide blot tests and peptide specific affinity chromatography (see 'Materials and methods' section). Most crucial, however, would be an analysis of their specificity using western blots containing fractionated protein extracts of recombinant UTY- and UTX-fusion peptides from the same exon region produced after transfection in *Escherichia coli* and specific IPTG induction (for details see 'Materials and methods' section). Blotting these protein extracts in parallel after gel electrophoresis on nylon membranes, we can demonstrate that the UTY-1 antiserum indeed only detects the recombinant parental UTY-peptide sequence and not the UTX-fusion peptide containing the same exon region. Similar, the UTX-1 antiserum only detects the parental UTX peptide but not the UTY analogue (Fig. 1). We, therefore, provide evidence, that our polyclonal UTY-1 and
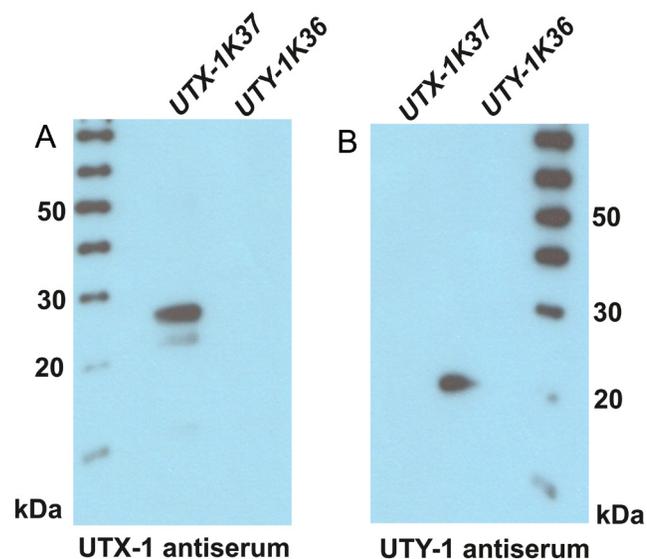
Figure 1 Western blot doublets with protein extracts of *UTX1K37* and *UTY-1K36* recombinants expressed in M15 (pREP4) cells after IPTG induction (60 min with 1 mM 1 mM IPTG). (A) First doublet stained for UTX with UTX-1 antiserum display cross-reaction only with the parental UTX-1 peptide expressed in *UTX-1K37*; (B) On the second doublet, the polyclonal UTY-1 antiserum only detects the corresponding parental UTY-1 peptide sequence cloned in the *UTY-1K36* recombinant. First lane in A and last lane in B present the Western Froxx all-in-one protein ladder fragments highlighting the 20, 30, 50 kDa marker fragments. For further description, see 'Materials and methods' section and main text.
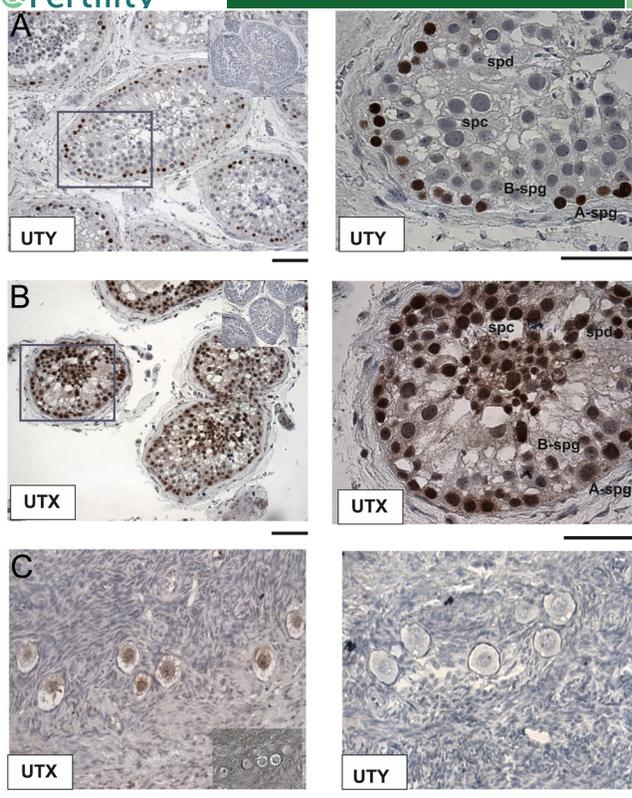
Figure 2 Immunohistochemical localisation of UTY and UTX protein expression in serial sections of human testes and ovarian tissue after staining with UTY-1 and UTX-1 polyclonal antisera, respectively. (A) UTY is expressed significantly only in A spermatogonia (A-spg) located at the basal membrane. See at the right section enlargement of the picture from the left marked with square (A-spg, A-spermatogonia; B-spg, B spermatogonia; spc, spermatocytes; spd, spermatids). (B) UTX expression is more spread in pre-meiotic male germ cells, and in round spermatids. See section enlargement at the right of picture from the left marked with square (abbreviations used are described in A). No reaction was found in control tissue sections of the same samples incubated with UTY-1 and UTX-1 pre-immune rabbit sera, respectively (see inserts in pictures at the left in A and B, respectively). (C) In the ovarian tissue section, only UTX expression is visible in the primordial female germ cells as expected (on the left). Lack of UTY expression in serial sections of the same tissue (on the right) confirms the specificity of both antisera for only their parental proteins UTX and UTY, respectively. Insert on the left: UTX picture display incubation of the same ovarian tissue section with the pre-immune rabbit serum of UTX-1. No positive staining reaction can be documented. Scale bars: 50 μm.

UTX-1 antisera mark the expression of only human UTY or UTX proteins, respectively.

UTY and UTX proteins are expressed in human germ cells

We first now wanted to explore with these UTY-1 and UTX-1 polyclonal antisera, where UTY and UTX proteins are expressed in human male germ cells after puberty. Immunohistochemical experiments on serial tissue sections of human testes with normal spermatogenesis revealed expression of UTY in A spermatogonia located

predominantly at the basal membrane (Fig. 2A). UTX is expressed comparable in these pre-meiotic male germ cells. However, the associated UTX-1 staining pattern suggests that UTX expression is more spread in the distinct types of spermatogonia, and additionally expressed in spermatocytes and round spermatids (Fig. 2B). In order to confirm the specificity of the observed UTY and UTX germ cell expression, we also analysed the expression of both proteins in some ovarian tissue sections (Fig. 2C). We only found a significant staining pattern with the UTX-1 antiserum marking UTX expression in the primordial female germ cells of the follicles, but no UTY-1 staining pattern. This confirms that both antisera detect expression of only their parental UTX and UTY proteins, respectively.

UTY and UTX are expressed during the development of foetal male germ cells

Expression of AZFa candidate gene, *UTY*, in human spermatogonia after puberty displays a similar pattern to that described earlier for *DDX3Y*, the major AZFa gene (Ditton *et al.* 2004). This would confirm our basic assumption that UTY is part of AZFa functional for promoting the pre-meiotic male germ cells towards meiosis (Vogt *et al.* 2007). To collect further experimental, we also analysed the expression of UTY and UTX in immunohistochemical experiments with the polyclonal UTY-1 antiserum proven to detect only UTY proteins (Figs 1 and 2) also on fetal testis tissue sections and compare its staining pattern with that of UTX-1 antiserum.

UTY expression was first found in fetal germ cells at the 16th gestation week (gw) (Fig. 3A). No UTY expression was found in fetal germ cells at the 15th gestation week. At 16th gw the fetal gonocytes are male determined and begin maturing asynchronously towards pre-spermatogonia also called T1 spermatogonia (Gueler *et al.* 2012). UTX expression seems to be lower than that of UTY at this foetal germ cell phase; its staining pattern is difficult to distinguish from that of the pre-immune-serum analysed in parallel. However, some weeks later UTY and UTX expression looks comparable (Fig. 3B: 25 gw; 3C: 33 gw). At the 41 gestation week, we could observe only UTY expression, whereas staining with UTX-1 displays only some intercellular background staining also observed with the pre-immune-serum (Fig. 3D).

We can conclude that the pattern of UTY expression in human foetal male germ cells is comparable to that of DDX3Y described earlier (Gueler *et al.* 2012). Both encoded proteins are expressed predominantly in spermatogonia; their loss should therefore interfere with the pre-meiotic
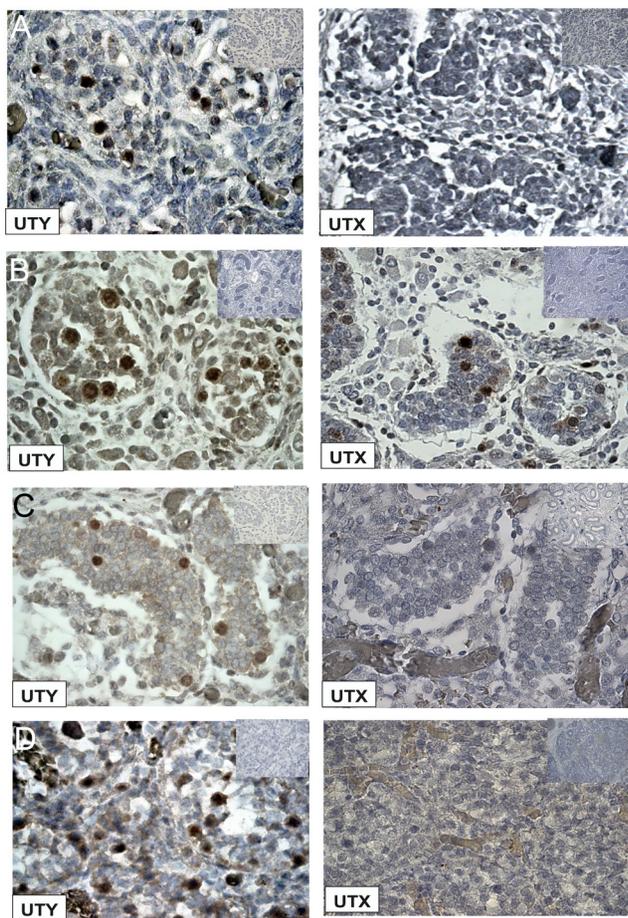
Figure 3 Immunohistochemical staining with UTY-1 and UTX-1 antisera for comparative analysis of UTY and UTX protein expression during fetal male germ cells development. (A) UTY and UTX staining pattern in fetal germ cells at 16th gestation week (gw) display stronger UTY expression in these gonocytes. Some weeks later (B: 25 gw; C: 33 gw) UTY and UTX expression looks comparable. (D) At 41 gestation week only UTY expression could be observed, whereas UTX expression is significantly reduced and becomes comparable to the background level (see also pre-immune staining pattern at this germ cell phase: insert at the right). Control tissue sections incubated with the UTY-1 and the UTX-1 pre-immune rabbit serum (see inserts at the right in each picture) display no specific staining patterns. Scale bar length for 50 µm shown on the right at D is the same in each picture displayed here.

human male germ cell development. The *UTY* gene is, therefore, most likely another functional part of AZFa as suggested earlier (Wimmer *et al.* 2002).

Comparison of UTY and DDX3Y expression in germ cell nests of females with complete gonadal dysgenesis (CGD) presenting gonadoblastoma or dysgerminoma pathology

AZFa gene, *DDX3Y*, was found recently to be part of the Gonadoblastoma susceptibility Y (GBY) locus overlapping with AZFa in proximal Yq11 (Vogt *et al.* 2019). We,

therefore, wanted to know whether GBY also includes the *UTY* gene. For this purpose, we compare the staining pattern of UTY-1 (marking expression of only UTY, see above) with that of DBY-10 (marking expression of only DDX3Y proteins; see Ditton *et al.* 2004) in serial gonadal tissue sections of two DSD-XY individuals with complete gonadal dysgenesis (CGD: Swyer syndrome). One sample (GBX88) presents in some of the undifferentiated gonadal tissue regions local germ cell nests with Gonadoblastoma (GB) pathology. One sample (GBY121) was found with dysgerminoma cells spreading in the undifferentiated gonadal tissue regions. GB cells are pre-malignant precursor cells developing towards germ cell tumour cells. These are designated as dysgerminoma when surrounded by granulosa cells marked by FOXL2 expression (Buell-Gutbrod *et al.* 2011).

We found comparable staining patterns with DBY-10 and UTY-1, marking comparable expression of DDX3Y and UTY in the gonadoblastoma cells of GBY88 in all tissue sections analysed (Fig. 4A). A similar strong expression of both proteins was also found in the dysgerminoma cells of GBY121 (Fig. 4B). We can thus conclude that the GBY tumour susceptibility locus on the long arm of the human Y chromosome, as presented in more detail earlier (Vogt *et al.* 2019), most likely includes also the *UTY* gene.
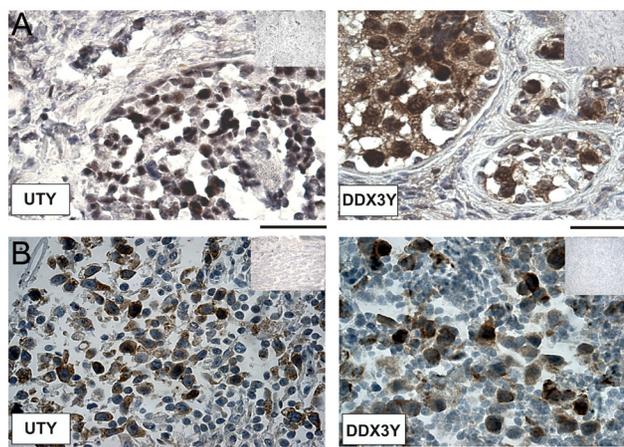
Figure 4 Immunohistochemical staining pattern with UTY-1, respectively, DBY-10 marks UTY, respectively, DDX3Y expression on serial tissues sections of two DSD-XY individuals with Swyer syndrome during tumour development. (A) GBY88 patient (16 years age) has developed pre-malignant gonadoblastoma cells in local germ cell nests of the undifferentiated gonadal tissue regions. Strong UTY and DDX3Y expression mark the gonadoblastoma pathology as described earlier (Vogt *et al.* 2019). (B) GBY121 patient (29 years age) has already developed dysgerminoma tumour cells from the pre-malignant gonadoblastoma cells. Again UTY and DDX3Y expression is comparable. It suggests that the genetic complexity of GBY tumour susceptibility locus in proximal Yq11 also includes the UTY gene. Scale bars: 50 µm.

Discussion

UTY and UTX is functioning in human male germ cells

Expression of UTY and UTX in human spermatogonia suggest that both histone 3 demethylase enzymes are involved in the H3K27 methylation/demethylation mediated chromatin modulation process observed during spermatogonia proliferation and differentiation from the spermatogonial stem cells (Tseng *et al.* 2015). Restriction of UTY expression in human A spermatogonia located at the basal membrane suggests that UTY supports UTX function also expressed in these cells probably including also the spermatogonia stem cell (SSC) fraction (Izadyar *et al.* 2011).

Other histone lysine methylases (KTMs) and -demethylases (KDMs) are reported to be involved later during male germ cell development; namely in monitoring the pre-meiotic chromosome pairing processes by silencing unpaired chromosomes, respectively, in controlling the activation of some specific spermatid genes (Nottke *et al.* 2009). Thereby LSD1/KDM1 and JARID1/KDM5 act on demethylation of H3K4me2/1, respectively, on H3K4me3/2 at zygotene/pachytene stage (Godmann *et al.* 2007) and JHDM2A/KDM3A mediates the demethylation of H3K9me2 in late pachytene and round spermatids (Okada *et al.* 2007).

In the mouse germ line, it has been shown that *Uty* is required to support the function of its paralog *Utx* located in the chromatin domains of primordial germ cells (PGC). Utx expression activates genes in these precursor germ cells by H3K27me2/3 demethylation to support their further differentiation programme (Mansour *et al.* 2012). In mouse somatic cells, the *Uty* gene encodes a male--specific H-Y epitope (H-YD[b]) of the H-Y antigen locus expressed during early embryogenesis (Greenfield *et al.* 1996). Accordingly, in human somatic cells, UTY also expresses a specific H-Y antigen (Warren *et al.* 2000). It has therefore been argued that UTY proteins are probably only required for some male cell specific functions being restricted to male-specific organs, such as the prostate (Dutta *et al.* 2016). However, *UTY* deletions seem not be associated with somatic pathologies although the genes transcripts and proteins were found to be expressed in multiple human tissues and in leukocytes (Lahn & Page 1997; www.uniprot.org/uniprot/O14607).

In human, the cellular level of *UTY* and *UTX* expression indicates some gender-specific dosage control function (Bellott *et al.* 2014), and it has been shown that the dosage of their expression is dependent on the genes copy number (Raznahan *et al.* 2018). Increased levels of UTY expression are associated with a 50% increased risk to suffer from a coronary artery disease, especially when the Y chromosome of these men is from Y haplogroup 'I' (Bloomer *et al.* 2013). Accordingly, there should be a cellular balanced UTX and UTY expression profile that can compensate each other. However, UTY is reported to have only a low enzymatic histone 3 K27 demethylase activity compared to UTX (Walport *et al.* 2014). Consequently, the efficiency of the UTY histone demethylase function is lower than that of the UTX protein and UTY expression is not as effective as UTX expression to suppress tumour development (van Haaften *et al.* 2009).

A major question would then be whether UTY and then probably also the UTX protein are only functional as H3K27me3 demethylase enzymes in the pre-meiotic male germ cells, respectively, whether both proteins rather serve for a histone demethylase independent function to control distinct germ cell genes required in human spermatogonia for balancing the spermatogonia proliferation and differentiation rates from the SSC pool after puberty.

The histone demethylase independent function of UTX and UTY is probably based on their conserved N-terminal tetratricopeptide repeats (TPRs), which is not present in the third KDM6 protein member, JMJD3 (Walport *et al.* 2014). These TPR motifs are strongly conserved from yeast to human as functional important protein interaction motifs (D'Andrea & Regan 2003). Accordingly, it has been shown that both paralogues do have similar functional roles in human metabolism and during development independent from their histone demethylase activity and these might even be co-regulated. Indeed, in mouse, both genes displayed a common pattern of transcription factors binding to the *Utx* and *Uty* promoters (Gazova *et al.* 2019).

It is, therefore, most likely, that UTY and probably then also UTX also express some demethylase independent function(s) in human pre-meiotic male germ cells after puberty.

During foetal male germ cell development, we observed UTY and UTX expression visible starting around the 16th gestation week (Fig. 3A). No staining pattern with UTY-1 and UTX-1 antibodies could be observed on foetal gonads tissue sections of the 15th gestation week (data not shown). Interestingly, whereas UTY expression was found consistently along foetal germ cell development, UTX expression looks variable and comparable to UTY only at the 25th gestation week (Fig. 3B). Significant lower UTX expression was found at the 16th and 33th gestation week; the UTX-1 staining pattern became rather visible in our immunohistochemical experiments although performed

in parallel with the specific UTY-1 and UTX-1 antisera in serial sections of the same foetal gonad specimens (Fig. 3A and C). No UTX expression could be detected at the 41st gestation week (Fig. 3D).

Stronger than UTX and consistent UTY expression in foetal spermatogonia would support our assumption of the functional importance of this Y gene together with the neighboured *DDX3Y* gene just for foetal male germ line development during their proliferation phase (Gueler *et al.* 2012). This also would fit to our observation that during adult spermatogonia development, UTY expression is concentrated in the proliferating A spermatogonia fraction located at the basal membrane (Fig. 2A).

Human UTY is part of AZFa locus on human Y chromosome

The genomic neighbourhood of *UTY* and *DDX3Y* genes on the human Y chromosome is conserved on the Y chromosome of distinct mammalian lineages including rodents and primates (Murphy *et al.* 1999, Wimmer *et al.* 2002, Li *et al.* 2013). This has suggested a conserved function of both proteins in the male germ line of these mammals. Accordingly, it has been designated as 'AZFa locus' (Wimmer *et al.* 2002, Vogt *et al.* 2007).

In this paper, we present strong experimental evidence that the proposed AZFa candidate gene, *UTY*, is involved in the control of the proliferation and differentiation process of foetal and adult spermatogonia, like the major AZFa gene, *DDX3Y*; both are predominantly expressed in spermatogonia located at the basal membrane (for DDX3Y see: Ditton *et al.* 2004, Gueler *et al.* 2012, Ramathal *et al.* 2015). Accordingly, *DDX3Y* deletions are reported to cause a severe pathology in the male germ line leading eventually to complete absence of all pre-meiotic germ cells (Foresta *et al.* 2000, Vogt *et al.* 2017). However, in some cases, patients with complete AZFa deletions, including the *DDX3Y* – but not the *UTY* gene – still display some spermatogonia in their testis tubules, suggesting that the complete germ cell aplasia observed in most AZFa patients may be probably only a secondary and age-dependent consequence of the primary mutation defect, namely, reduction of the spermatogonia proliferation rate (for further discussion see also Vogt *et al.* 2017, 2008). Most interestingly, like somatic *UTY* deletions, also *DDX3Y* deletions do not cause any somatic pathology in these infertile men, suggesting that DDX3Y like UTY function is restricted to the male germ line.

In one patient with complete absence of all germ cells in his testis tubules, we found a distal extension of the classical AZFa deletion interval, including additionally the *UTY* gene (Vogt *et al.* submitted). Not any spermatogonia were found in the patient testis tubules, presenting a complete Sertoli-Cell-Only (SCO) syndrome. Based on the germ cell UTY expression pattern presented in this paper (Figs 2 and 3), we may therefore speculate that infertile men with deletion of both these Y genes in AZFa, *DDX3Y* and *UTY*, indeed strengthens the primary testicular phenotype of the 'AZFa deletion' pathology, that is, disruption of spermatogonia proliferation cycle. DDX3Y expression is known to be functional during the G1-S phase of the cell cycle (Ditton *et al.* 2004, Ramathal *et al.* 2015).

However, to be cautious: until we have not found also some single *UTY* deletion/point mutations on the Y chromosome of infertile men with SCO, it is not yet proven, whether there is also some functional link between UTY and DDX3Y expression in the same pre-meiotic human male germ cells.

UTY is part of the GBY susceptibility locus in proximal Yq11

We have recently shown that *DDX3Y* and *TSPY*- is part of the Gonadoblastoma Locus of the human Y chromosome (GBY) expressed in the aberrant male germ cells of women with some disorders of sexual development (DSD) and a Y chromosome in their karyotype (DSD-XY individuals; Vogt *et al.* 2019). In this paper, we now present experimental evidence that UTY probably also contribute to this GBY tumour susceptibility function. In this paper, we analysed UTY expression in the aberrant male germ cells of the dysgenetic gonads of two DSD-XY individuals with complete gonadal dysgenesis (Swyer syndrome) when developing gonadoblastoma, respectively, dysgerminoma cells (Fig. 4). UTY expression was found to be comparable to that of DDX3Y in the dysgenetic gonads of both patients, which do have still some germ cell nests in their streak gonads. The molecular origin for their high risk to develop gonadoblastoma and further dysgerminoma is generally based on their phenotype of pluripotency marked by OCT3/4 expression (Cheng *et al.* 2007; for further details, see Cools *et al.* 2009, Vogt *et al.* 2019). Our data confirm the basic assumption discussed earlier that probably all AZFa Y genes functional for the pre-meiotic proliferation-differentiation balance of spermatogonia during male germ cell development support tumour development in dysgenetic female gonads because of some dysregulation in their aberrant germ cells.

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