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COMPLEMENTARY EXPRESSION AND PHOSPHORYLATION OF Cx46 AND Cx50 DURING DEVELOPMENT AND FOLLOWING GENE DELETION IN MOUSE AND IN NORMAL AND ORCHITIC MINK TESTES

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Short title: Cx 46 and Cx50 gene transcription and distribution in testis

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ABSTRACT

Gap junction-mediated communication helps synchronise interconnected Sertoli cells activities. Besides, coordination of germ cell and Sertoli cell activities depends on gap junction-mediated Sertoli cell-germ cell communication. This report assesses mechanisms underlying the regulation of Cx46 and Cx50 in mouse testis and those accompanying a “natural” seasonal and a pathological arrest of spermatogenesis resulting from autoimmune orchitis (AIO) in mink. Furthermore, the impact of deleting Cx46 or Cx50 on the expression, phosphorylation of junction proteins and spermatogenesis is evaluated. Cx46 mRNA and protein expression increased while Cx50 decreased with adulthood in normal mice and mink. Cx46 mRNA and protein expression increased while Cx50 decreased with adulthood in normal mice and mink. During the mink active spermatogenic phase, Cx50 became phosphorylated and localized to the site of the blood-testis barrier. By contrast, Cx46 was dephosphorylated and associated with annular junctions suggesting phosphorylation/dephosphorylation of Cx46 and Cx50 involvement in the barrier dynamics. Cx46-positive annular junctions in contact with lipid droplets were found. Cx46 and Cx50 expression and localization were altered in mink with AIO. Deleting Cx46 or Cx50 impacted on other connexins expression and phosphorylation and differently affected tight and adhering junction protein expression. The level of apoptosis determined by ELISA and number of Apostain-labelled spermatocytes and spermatids/tubule were higher in Cx46-/- than WT and Cx50-/- mice arguing for a life-sustaining Cx46 gap junction-mediated exchanges in late-stage germ cells secluded from the blood by the barrier. The data show that expression and phosphorylation of Cx46 and Cx50 is complementary in seminiferous tubules.

Key words: Sertoli cell, lipid droplet, spermatogenesis, meiosis, seasonal breeder
INTRODUCTION

The seminiferous epithelium is unique in that it holds somatic Sertoli cells that divide by mitosis and germinal cells that divide first by mitosis then by meiosis before differentiating into spermatids unlike most epithelia in the body that bear a single type of cells that only undergo mitosis. The survival of the two distinct cell types is contingent upon successful Sertoli cell and germ cell physiological symbiotic activities where the function of one cell type impacts the function of the other and reciprocally. The establishment of occluding zonules joining adjacent Sertoli cells seals the paracellular route establishing a physical barrier that creates and separates two superposed cellular compartments within the seminiferous epithelium (27; 77). Residing in the basal compartment situated inferiorly to the barrier of the tubule and adjacent to the limiting membrane, the early-stage germ cells benefit from a direct access to blood-borne substances. The late-stage germ cells dwelling the lumenal compartment situated superiorly to the barrier are in contrast forbidden direct vascular exchanges (17; 72; 76). This particular distribution of the so-called blood-testis barrier within the seminiferous epithelium constrains the late-stage germ cells to rely on gap junction-mediated communication with adjoining Sertoli cells to carry out functions vital to their own survival yet unresolvable to germinal cells alone (73-75).

The gap junctions are made up of multimeric channels individually built of transmembrane segments of proteins: the connexins (54; 63) that belong to a multigene family (105). The contacting cells each contribute one homomeric or heteromeric hemichannel to the cell surface. Since most cells express more than one connexin, the pairing of hemichannels may result in homotypic or heterotypic gap junctional channels that assemble into junctional plaques joining cells (40). Because the unitary conductance of gap junctions is connexin-dependent, the prevalence
of one or more connexin species will impose a specific permeability to the cells at times favouring
at others precluding the passage of regulatory molecules between cells (14; 40). In the context of
spermatogenesis, the molecular diversity (10; 101), the phosphorylation status and turnover of
connexins (43) greatly impact gap junction-mediated Sertoli cell-to-Sertoli cell and Sertoli cell-to-
germ cell channels and communication (75; 99). The selective intercellular traffic of regulatory
molecules will condition cellular growth, differentiation and death in the seminiferous epithelium.

The lack of a direct access to the blood supply is not restricted to the late-stage germ cells
of the seminiferous epithelium. In the naturally avascular lens of the eye, the ionic and water
balance of the intercellular milieu, transparency and optical properties are conditioned by gap
junction-mediated communication (58). Cx43 is primarily confined to the anterior epithelial
monolayer of the lens in contrast to Cx46 and Cx50 that are co-expressed in the fibre cells of the
nuclear more central region of the lens where they share the same junctional plaques (26; 67).
Because of the similarity of the lens fibre cells with the late-stage male germ cells in that they both
lack direct access to the blood supply, the expression, localisation and interaction of Cx46, Cx50
and Cx43 amongst themselves and with other junction proteins was examined in the seminiferous
epithelium. The present study is the first to evaluate the physiological significance of Cx46 and
Cx50 on the outcome of spermatogenesis. This study aims to establish whether the regulation of
the expression and phosphorylation of Cx46, Cx50 and Cx43 is synchronous or complementary in
the seminiferous epithelium. We tested if individually altering genes coding for either Cx46 or
Cx50 impacts on the expression of remaining junction proteins. The report also attempts to get an
insight into the mechanisms underlying the interaction between these connexins during postnatal
development in mouse and during a “natural” seasonal as well as a pathological arrest of spermatogenesis resulting from spontaneous autoimmune orchitis (AIO) in mink.

Novel evidence that the expression and phosphorylation of Cx46 and Cx50 is complementary not only during postnatal development in the mouse and mink seminiferous epithelium but also throughout the “natural” seasonal as well as the pathological arrest of spermatogenesis resulting from spontaneous autoimmune orchitis (AIO) in mink is presented. Moreover, we show that knocking out Cx46 results in an increase in 51kDa Cx50 band accompanied by a decrease in the phosphorylated 60kDa Cx50 band and increases Cx43 while decreasing occludin and N-Cadherin in the tubules. By contrast, deleting Cx50 causes a decrease in the phosphorylated 68kDa Cx46 and in ZO-1 accompanied by an increase in PCx43 (phosphorylated in serine 368), Cx43 and Claudin11. Furthermore, we found that the phosphorylation of Cx46 and Cx50 is opposite in the seminiferous epithelium. The results advocate for a complementary expression and phosphorylation of Cx43, Cx50 and Cx46 in the mouse and mink seminiferous epithelium. In addition, the data show that when Cx46 affects the tight junction and adhering junction proteins, Cx50 has no effect and vice versa. Our finding that Cx46-/- mice are characterised by increased numbers of Apostain-positive cells especially amongst spermatocytes and spermatids concurrent with increased intratubular apoptosis levels measured by ELISA offers evidence of a life-sustaining role of Cx46-mediated intercellular communication on late-stage germ cells secluded by the blood-testis barrier.
MATERIALS AND METHODS

Animals:

Mouse: The mouse model provides invaluable opportunities to explore consequences of altering the coding of specific genes on precise tissue functions. However, the small size of mouse testis can provide only limited quantities of seminiferous tubule-enriched fractions. Mice were first anaesthetized (urethane, 1 g/kg IP, Sigma, St-Louis, MO, USA) before being decapitated. Animal use protocols were approved by University of Montreal Animal Care Committee.

Normal Mouse: male mice were purchased from Charles River (St-Constant, QC, Canada) of the BALB/cJ background and were housed at room temperature with food and water *ad libitum* and exposed to a 12 h: 12 h light-dark cycle. Studies on development were carried out on mice aged from 7 to > 60 days (d) old or adult. Five animals were used per age group except for age 14-28d for which 10 animals were used due to the small size of pups’ gonads.

Cx46 and Cx50 knockout (KO) mice: The generation of homozygous mice lacking Cx46 (*Cx46*/*-) and Cx50 (*Cx50*/*-) has been described elsewhere (33; 85). Mice aged 16 weeks old were first anaesthetised then decapitated as stated above then their testes were removed and processed for different studies. *Cx46* +/+ and *Cx46*/*- mice were from 129SvJ strain while *Cx50*+/+ and *Cx50*/*- mice were from 129SvJ X C57BL/6J mixed strain background. Three male *Cx46*/*- mutant mice and three WT and three *Cx50*/*- mutant mice and three WT were used.

Mink: The annual reproductive cycle of the mink (*Mustela vison*) provides a unique opportunity to consider the impact of a “natural” seasonal and reversible arrest of spermatogenesis on the interactive regulation of Cx46-, Cx50- and Cx43-mediated gap junctions. Moreover we (74; 75;
82) and others (96; 97) have established the mink as a valuable model for the study of male reproduction and autoimmunity in a seasonal breeder. AIO develops spontaneously in 30% of the black fur-coated mink (82; 98). Similarly, in humans, spontaneous autoimmune orchitis (AIO) is characterized by a non-infectious granulomatous orchitis, spermatogenic arrest and, deposits of immunocomplexes in the basement membrane associated with infectious diseases resulting from systemic viral infection (47; 61; 89). Immunological male infertility (55) is associated with pathological features emulating granulomatous orchitis documented in mink with AIO (95) in which disruption of germ cell growth and Sertoli cell function results in aberrations in the processes of gene transcription and post-translational phosphorylation of Cx43(74; 75; 82). The use of the mink model provides the distinctive advantage of identifying the difference in the impact of a pathological versus a natural arrest of spermatogenesis on this regulation. Mink were purchased from Visonnière St. Damase (St. Damase, QC, Canada). They were individually caged with food and water at libitum and natural lighting. Animal received a 0.9 ml/kg body weight of Na-phenobarbital (Somnotol, MCI Pharmaceutical, Mississauga, ON, Canada) and 0.15ml/kg of a solution of 0.3g/ml chloral hydrate. The right testis with its epididymis was used in microscopy and immunohistochemistry studies, the left in tissue fractions. The experiments were conducted in accordance with the Université de Montréal animal care committee.

*Normal mink:* The testes from a total of 135 mink were used: 10 neonatal, 10 pubertal and 5 adult per month. Harvesting was done at thirty-day intervals from 90- to 270 day after birth. Tissues were collected from 2-3-year-old fertile adults the last week of each month during the annual reproductive cycle.
Infertile mink with AIO Two to 3-years old Black and Sapphire (genetically related to Black) mink that mated and sired 5 or more litters the previous year but were sterile during the current year and diagnosed with secondary infertility related to spontaneous AIO (98) were utilised. Criteria of fertility The morphology, motility and number of spermatozoa obtained from the ejaculated semen recovered from vaginal lavage was assessed under the light microscope in March. Anti-sperm antibody levels in serum were measured (3; 82). A total of 20 mink with low sperm counts or immobile spermatozoa, high antibody levels and whose histopathology showed leucocyte infiltration and destruction of seminiferous tubules at autopsy and diagnosed with secondary infertility were used in this study: 10 in February and 10 in March.

Chemicals: Phenylmethane-sulfonyl fluoride (PMSF), leupeptin, aprotinin, cell death detection ELISA kits and Lumi-lightPlus chemiluminescence detection kits were purchased from Roche (Laval, QC, Canada). Potassium bisperoxo (1, 10-phenanthroline) oxovanadate (V) [bpV (phen)] was obtained from Calbiochem (San Diego, CA, USA) and the cell death detection ELISA kit from Boehringer-Mannheim (Laval, QC, Canada).

Antibodies: The following antibodies were used: Rabbit polyclonal anti-caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), purified mouse anti-flotillin-1 (BD Biosciences, Mississauga, ON, Canada), monoclonal Ab against single-stranded DNA F7-26 (Apostain) (Alexis; San Diego, CA, USA), rabbit polyclonal anti-Cx46 (alpha-Diagnostic International, San Antonio, USA), mouse monoclonal IgM anti-Cx50 (In Vitrogen Canada Inc., Burlington ON, Canada), rabbit polyclonal anti-N-cadherin and rabbit polyclonal anti-claudin-11 (Abcam,
Cambridge, MA, USA), rabbit polyclonal anti-occludin (Zymed Laboratories, San Francisco, CA, USA), rabbit polyclonal antibody raised in rabbits against a synthetic peptide corresponding to the amino acid sequence 363-382 of human Cx43 that recognizes all Cx43 forms (Pan-Cx43 antibody) and monoclonal myosin light chain (MLC) antibody (Sigma, St-Louis, MO, USA); a rabbit polyclonal antibody recognizing Cx43 phosphorylated in serine 368 (P-Cx43) and developed against a synthetic phospho-peptide corresponding to residues surrounding Ser368 of human Cx43 (Chemicon, Temecula, CA, USA); HRP-conjugated streptavidin (Molecular Probes, Eugene, OR, USA); horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG, HRP-conjugated anti mouse IgM, biotinylated anti-rabbit F(ab’)2 and biotinylated anti-mouse F(ab’)2, were obtained from Jackson Immunoresearch Laboratories (Mississauga, ON, Canada).

**Isolation of seminiferous tubule-enriched fractions**

Distinct anatomical and functional characteristics set apart the interstitium and the seminiferous tubules of the testis. Yet, today most assays remained carried on whole testis extracts rather than on interstitium- and/or seminiferous tubule-enriched fractions as we have done here. We have showed that a beforehand exposure to even a mild enzymatic digestion hinders the detection of phosphorylated and/or glycosylated forms of proteins in the samples (2). For this reason, here, the seminiferous tubule-enriched fractions were obtained without a pre-digestion to preserve the integrity of the proteins in the samples using a technique we described elsewhere (2). Briefly, the seminiferous tubules were teased apart from the interstitium from freshly decapsulated testes on ice in phosphate buffered saline (PBS: 137mM NaCl, 3mM KCl, 8mM Na2HPO4, 1.5mM KH2PO4, pH 7.4) containing 2 mM PMSF, 1 mM EGTA, 2 :g/ml leupeptin, 2 :g/ml aprotinin,
4 mM Na3VO4, 80 mM NaF and 20 mM Na4P2O7 with 10 μM bpV (phen)). The seminiferous tubule-interstitium solution was allowed to decant then centrifuged 15 min at 400 rpm, (GS-6R Beckman Centrifuge, JH-3.8 Rotor) at 4°C. The interstitium (ITf) and the seminiferous tubule-enriched (STf) fraction were individually recovered after a centrifugation at 1000 rpm for 10 min (GS-6R Beckman Centrifuge, JH-3.8 Rotor) at 4°C and characterised under the microscope.

**Isolation of lenses**

Lenses were excised from adult normal mice and mink and put on dry ice for fragmentation. After thawing, the fragments were homogenized in PBS containing protease and phosphatase inhibitors using a Polytron PT 3100 homogenizer.

**Protein quantification** Proteins in samples were assayed using the method of Bradford (11) and materials from BioRad (BioRad, Mississauga, ON, Canada).

**Electrophoresis and Western blot analyses** Twenty to thirty μg total homogenate of each sample were loaded on polyacrylamide gels, separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes and subjected to Western blotting as previously described (75; 82; 99). Briefly, membranes were blocked 1 h at 37°C with 5% skim milk in Tris-buffered saline (TBS, 137 mM NaCl, 27 mM KCl, 25 mM Tris-HCl pH 7.4) then incubated with the different antibodies: Pan-Cx43 antibody (1:20,000), P-Cx43 (1:400), anti-caveolin-1 (1:3,000), anti-flotillin-1 (1:250), anti-Cx46 (dilution 1:500), anti-Cx50 (dilution 1:25), anti-N-cadherin (1:1,000 dilution), anti-occludin (1:3,000 dilution), anti-claudin-11 (1:1,000 dilution). The antibody dilutions were prepared in 5%
skim milk-TBS. Membranes were washed in TBS containing 0.05% Tween 20 and incubated 1h with a corresponding secondary antibody conjugated to HRP at room temperature. The antigen-antibody complexes were detected by chemiluminescence. The density of each antibody bands was quantified by laser scanning with the public Scion Image Software (Scioncorp, MD, USA).

**Alkaline phosphatase treatment** Fifty Φg of proteins of STf homogenate were incubated with 10-15 units of calf intestine alkaline phosphatase (Boheringer Mannheim, Germany) in 30 Φl of digestion buffer (10mM MgCl₂, 1mM ZnCl₂ 50mM Tris-HCl pH 8.0) for 2hs at 37°C in the presence or absence of phosphatase inhibitor. The reaction was stopped by incubating the samples with 10% trichloroacetic acid for 30 min at 4°C. Pellets were washed in cold acetone, dissolved in sample buffer and boiled for 3 min. Thirty Φg proteins were applied per lane separated by SDS-PAGE and exposed to Western blotting with antibodies.

**Preparation of lipid raft-enriched membrane fractions** Lipid raft/caveolae-enriched membrane fractions were purified according to a variation of the method described by Schubert et al., 20012 (91) as follows. Tubule-enriched fractions from adult >60 days old mouse and mink obtained in February and in November were washed in cold PBS and homogenized in 750 :l of morpholinoethanesulfonic acid (MES)-buffered saline (MBS, pH 6.5) containing 1% Triton X-100 in a 5 ml ultracentrifuge tube. Each sample was mixed with an equal volume of 80% sucrose (prepared in MBS without Triton X-100), and overlaid with a discontinuous sucrose gradient of 1.5 ml of 30% sucrose and 1.5 ml of 5% sucrose (prepared with MBS without Triton X-100). The samples were centrifuged at 4 °C for 18 h at 200,000g (Beckman XL-70 ultracentrifuge). The rafts
came out as a hazy whitish band floating at the 5-30% sucrose interface. Aliquots of each fraction were subjected to SDS-PAGE and immunoblotting.

**Real Time Quantitative Polymerase Chain Reaction (Real time Q-PCR)** Total RNA was isolated from the tubule-enriched fractions using Trizol (Life Technology, Grand Island, NY, USA) as per the manufacturer’s instructions. After isolation, 1 μg of total RNA was reverse transcribed into cDNA at 42°C for 1 h using Oligo (dT) and 1 μl of iScript Reverse transcriptase (iScriptTM cDNA Synthesis kit, Bio-Rad, Hercules, CA, USA) in a 20 μl final volume. The mRNA expression of Cx46, Cx50 and Cx43 was measured using primers designed for mouse or human by Primer 3 software (Premier Biosoft International, Palo Alto, CA, USA) (Table 1). The primers were purchased from Invitrogen Corp. (Invitrogen, Carlsbad, CA, USA). The real time quantitative PCR (real time Q-PCR) reactions were performed using iQTM SYBR Green Supermix according to the manufacturer’s instructions and 0.5 μM of each primer. In all real time Q-PCR reactions, a negative control corresponding to RT reaction without the reverse transcriptase enzyme and a blank sample were carried out (data not shown). Amplification of the housekeeper gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control to quantify the expression of a given gene in real time Q-PCRs.

**Preparation of tissues for immunolocalization of Cx46, Cx50 and Aposatine** Testes were perfusion-fixed with Bouin’s fixative (71; 79). Endogenous peroxidase activity was inhibited with 0.6% hydrogen peroxide (H₂O₂) in 70%ethanol (80). Unspecific binding was blocked with 0.5% skim milk in TBS containing 0.1% Tween-20 (TBSTw) for 1h at 37EC. Next, tissue sections were
incubated with the different antibodies using the method described elsewhere (80). The tissue sections were washed in TBST after each incubation and exposed to TBS containing 0.01% H$_2$O$_2$, 0.05% diaminobenzidine tetrachloride (pH 7.7) for 10 min at RT (37). The sections were washed in water and counterstained in a 0.05% aqueous methylene blue, mounted in Permount and examined on a Carl Zeiss Axiophot 2 fluorescence microscope. The stage-dependent distribution of the different antibodies was evaluated using the identification method of the stages of the cycle of the seminiferous epithelium of Pelletier in mink (68).

**Electron microscopy** Testicles were perfused through the testicular artery first with PBS (154 mM NaCl, 100 mM Na phosphate pH 7.3) followed by a diluted mixture of Karnovsky’s fixative (38) 0.1M Na cacodylate buffer pH 7.3 then processed as previously described (69). Briefly, tissues were post-fixed in an aqueous solution of 1% osmium tetroxide-1.5% potassium ferrocyanate, processed in a 1% solution of tannic acid buffered to pH 7.8 with 0.1M Na cacodylate and stained en bloc in a solution of 5% uranyl acetate in Veronal acetate buffer pH 4.5-5.2, dehydrated in ethanol and embedded in PolyBed 812 (PolySciences Ltd, Warrington, PA, USA). Silver-to-gold thin sections collected on formvar-coated carbon stabilized grids were examined at 80kv with a Philips 300 electron microscope.

**Freeze-fracture replication** Aldehyde-fixed testes were washed in 0.1M Na cacodylate buffer pH 7.3, cryoprotected in a solution of 30% glycerol in 0.2M Na cacodylate, pH 7.35 (69; 70; 77) and fractured in a BAF Balzer apparatus at-115EC. The replicas were collected on formvar-coated carbon stabilized grids and examined at 80kv with the electron microscope.
**Cholesterol labelling with filipin** Mapping cholesterol with filipin (Polysciences, Warrington, PA, USA) was achieved as described earlier (78; 81).

**Data and statistical analysis** Analyses were done with Stata software (Stata Corporation, College Station, TX, USA). The data were evaluated with the Student’s *t* test or the analysis of variance (ANOVA) followed by the Tukey HSD test.
RESULTS

Characterisation of the seminiferous tubule-enriched fractions

The seminiferous tubule-enriched fractions (STf) were obtained without enzymatic digestion. The rationale for our using this approach to generate the tubule fractions was based on our earlier report that in comparison to other approaches that use a beforehand enzymatic digestion, avoiding this step preserves the integrity of proteins in the samples under study allowing detection of otherwise undetectable isoforms while yielding high amounts of relatively pure fractions (2). Furthermore, the characterisation of tubules fractions resulting from the use of our approach under the light microscope reveals that the cellular elements contained are apparently well preserved. All biochemical analyses in this study have been realised on seminiferous tubule-enriched fractions obtained without the use a beforehand enzymatic digestion and as previously described (2).

Cx46-, Cx50- and Cx43-mRNA in mouse during development

The Cx46 mRNA levels roughly doubled from 14 to 21 days after birth then increased further by adulthood (>60 days old) in mouse seminiferous tubule-enriched fractions (Fig. 1A). The profiles of tubular Cx50 and Cx43 mRNA levels were opposite to that of the Cx46 mRNA levels during the same time period. In contrast to Cx46 mRNA levels, Cx50 mRNA levels were highest by 14 days after birth then, significantly dropped five folds by 21 days before stabilising after 60 days (Fig. 1A). The Cx43 mRNA profiles emulated Cx50 mRNA levels by being elevated 14 days after birth then, significantly falling four folds with the completion of spermatogenesis in >60 days old mice (Fig. 1A).
**Cx46 and Cx50 in lens and seminiferous tubule-enriched fractions**

The ocular lens which expresses both Cx46 and Cx50 (103) was used as positive control (Fig. 1B). The Cx46 antibody detected an intense 51kDa band in both mouse (Fig. 1B) and mink (results not shown) lenses. In the seminiferous tubule-enriched fractions (STf) of both species, the 51kDa band appeared flanked by a heavier 68kDa band (Fig. 1B).

The Cx50 antibody detected a band around 51kDa and another around 60kDa in mouse (Fig. 1B) and mink (result not shown) lenses. The two bands were also detected in the seminiferous tubule-enriched fractions of both species (Fig. 1B).

**Phosphorylation status of the Cx46 and Cx50**

To determine the phosphorylated status of the Cx46 and Cx50 immunoreactive bands identified above (Fig. 1B), tubule-enriched fraction samples were incubated in buffer alone or containing alkaline phosphatase (Ph) in either the absence (-) or the presence (+) of phosphatase inhibitor (I) (Fig. 1C). After treatments, the samples were subjected to SDS-PAGE then probed with either Cx46 or Cx50 antibodies. The intensity of both the 68- and 51kDa Cx46 immunoreactive bands decreased after treatment (Fig. 1C). The results indicate that the 68kDa band contains phosphorylated forms of Cx46. This notwithstanding, that the 51kDa band contains phosphorylated forms of Cx46 besides the non-phosphorylated Cx46 cannot be ruled out. The apparent decrease in the 51kDa band’s intensity suggests that Cx46 may be rapidly degraded upon dephosphorylation. Alkaline phosphatase treatment decreased the intensity of the 60kDa Cx50-immunoreactive band while increasing that of the 51kDa band (Fig. 1C), indicating that the 60kDa
band contains phosphorylated forms of Cx50. However, that the 51kDa band contains phosphorylated forms of Cx50 in addition to non-phosphorylated forms cannot be ruled out.

**Cx46, Cx50, Cx43, Occludin and Claudin 11 and lipid rafts**

Rationale: The high concentration of sphingolipids and cholesterol of the lipid rafts promotes the gathering or the exclusion constituents from microdomains (83) and controls the delivery, assembly and removal of individual proteins from selected membrane domains (44). Cx46- and Cx50-mediated gap junctions are enriched in cholesterol and sphingomyelin (4; 34) suggesting that connexin channels could segregate into lipid rafts. Furthermore, we have reported elsewhere that formation/dismantling of gap junctions arises in lipid-rich microdomains and that their maturation appears in cholesterol-poor domains (74; 76; 77; 81) advocating in favour of a sorting of connexin-mediated channels according to their species or pairing characteristics through differences in lipid/protein ratios in Sertoli cell membranes. At present, Cx43 has been reported to interact with caveolin-1 and caveolin-2 (45) in other cell systems while Cx46 and Cx50 has been documented in lens caveolae (49) but the presence or absence of these three connexins in lipid rafts of the testis has never been established. This study aims to test this. The information is of relevance because of the documented contribution of the rafts to intracellular signaling interactions in other cell systems (21).

Because tubule-enriched fractions were obtained in larger amounts from mink than mouse testes, the lipid rafts studies were carried out in mink. Detergent-insoluble glycolipid (DIG) microdomains were separated by sucrose-density centrifugation in twelve equal volume fractions (1 = top of the gradient, 12 = bottom of the gradient (pellet) ). The seminiferous tubule-enriched
fractions (STf) lysate was used as control. The fractions were collected then, first probed with different antibodies before being subjected to Western blotting (Fig. 1D). The STf caveolin-1 and flotillin-1 were both used to identify the DIG fractions (DIG = fraction 4; Fig. 1D). The gap junction proteins Cx46, Cx50 and Cx43 were recovered in the DIG fraction (Fig. 1D). As well, the tight junction proteins occludin and claudin 11 were recovered in the DIG fraction (Fig. 1D).

**Cx46, Cx50 and Cx43 protein expression during mouse development**

The studies begin at 14 days (d) after birth (Figs. 1E-1G) because before day 14 after birth, the size of the developing mouse testes is too small to yield relatively pure tubule-enriched fractions in satisfactory amounts.

**Cx46:** The intensity of both the 51- and ~68kDa Cx46 immunoreactive bands increased throughout development reaching highest levels by adulthood albeit the increase in the 68kDa occurred earlier than that of the 51kDa band (Fig. 1E). The 51kDa levels increased significantly by 35 days compared to 28 days then again by 42 days compared to 35 days after birth. The ~68kDa Cx46 immunoreactive band levels increased significantly by 21 days compared to 14 days as well as by >60 days compared to 21 days (Fig. 1E). A 14kDa band was observed to decrease with development when whole membranes were exposed (not shown) in contrast, to the 51- and 68kDa Cx46 immunoreactive bands that both increased throughout development.

**Cx50:** In contrast to Cx46, the 51kDa Cx50 immunoreactive band showed a decrease in intensity in adulthood (<60 days) (Fig. 1F). The 51kDa band levels progressively declined first from 28 to 35 days then, from 42- to >60 days (Fig. 1F). The 60kDa Cx50 immunoreactive band levels first significantly decreased from 14 to 21 days then significantly increased from 21 to 42
days then again decreased in >60 days (Fig. 1F). Together, these results show that the 51- and 68kDa Cx46 (Fig. 1E) and the 51kDa Cx50 levels varied in a complementary fashion in normal adult mice seminiferous tubule-enriched fractions (Fig. 1F).

**Cx43**: The total Cx43 levels measured using the Pan Cx43 antibody steadily and significantly decreased first from 14 to 21 days and again from 21 to 28 days (Fig. 1G). Total Cx43 levels reached lowest values by adulthood in mouse tubule-enriched fractions when compared to 14d values (Fig. 1G). Total Cx43 levels and the levels of the 51kDa Cx50 immunoreactive band both decreased by adulthood (Fig. 1F) by contrasts to the levels of the 51- and 68kDa Cx46 (Fig. 1E) that instead increased.

*Effects of knocking out Cx46 or Cx50 on other connexins*

**Cx46**: As expected, the 51kDa and 68kDa Cx46 immunoreactive bands both significantly decreased to trace levels in Cx46-/- mouse tubule-enriched fractions compared to WT (Fig. 1H’, Cx46). The 68kDa Cx50 immunoreactive band levels significantly decreased in Cx50-/- mice compared to WT (Fig. 1H’, Cx46).

**Cx50**: Similarly, no Cx50 immunoreactive bands were detected in Cx50-/- mice (Fig. 1H”, Cx50). The 51kDa Cx50 immunoreactive band levels more than doubled by contrast to the 60kDa Cx50 immunoreactive band levels that significantly decreased in Cx46-/- compared to WT (Fig 1H”, Cx50). Together, these observations evidence the interdependent complementarity in the regulation of the expression of these two connexins species within the seminiferous tubules.
Cx43: PCx43 (phosphorylated in serine 368) levels significantly increased in Cx50-/- compared to WT mice (Fig. 1I). Total Cx43 levels significantly augmented in both Cx46-/- and Cx50-/- mice tubule-enriched fractions compared to WT (Fig. 1I).

Effects of knocking out Cx46 or Cx50 on tight junction proteins

The occludin levels were significantly diminished while claudin 11 and ZO-1 levels showed little changes in Cx46-/- mice compared to WT (Fig. 1J). In contrast, occludin changed little, claudin 11 significantly augmented but ZO-1 decreased in Cx50-/- mouse tubule-enriched fractions compared to WT (Fig. 1J).

Effects of knocking out Cx46 or Cx50 on N-Cadherin

The N-Cadherin levels significantly diminished in Cx46-/- mice but not in Cx50-/- where they showed little changes compared to WT mice (Fig. 1J).

Together, the results suggest that in addition to an apparent complementarity in the expression of Cx46 and Cx50, silencing either Cx46 or Cx50 affected differently not only the expression of other connexins gap junction isoforms but also the expression of the tight and adhering junction proteins in mouse seminiferous tubules. Furthermore, the data show that when Cx46 affects the tight junction and adhering junction proteins, Cx50 has no effect and vice versa (Fig. 1I). Table 2 provides a summary of the compensatory effects of deleting Cx46 or Cx50 on remaining junction protein in the tubule fractions.

Cx46 and Cx50 in mink seminiferous tubule-enriched fractions
**Mink reproduction:** Mink (*Mustela vison*) breed in March in the Northern hemisphere. The female mink have a delayed implantation and pups are born in April-May (62). Figs 2A and 2B respectively depict a calendar of the germ cell’s population recorded monthly during mink postnatal development and annual seasonal reproductive cycle. Figure 2B shows the active spermatogenic phase is being followed by an inactive spermatogenic phase. The time periods when the blood-testis barrier is incompetent in blocking vascularly infused permeability tracers are identified by the shaded areas by contrast to the non-shaded areas that mark the periods when the barrier blocks entry of blood-borne content in seminiferous tubules (Figs 2A, B) (68; 74; 75; 82). We have presented strong evidence that the endowment of impermeability of this barrier coincides with the canalisation of a lumen in the seminiferous tubules not with the colonisation of the epithelium by a particular generation of germ cells (68; 74).

**Cx46-, Cx50- and Cx43 mRNA expression in normal mink**

*During development:* Cx46 mRNA levels increased steadily by 270 days in adulthood when the values recorded were significantly higher compared to 120 days old mink (Fig. 2C). In contrast, Cx50 mRNA levels significantly decreased by 270 days in comparison to 120 days old (Fig. 2C). Cx43 mRNA profiles emulated the Cx50 profiles by being highest by 120 days then abruptly dropping by 180 days and remaining low by 270 days (Fig. 2C).

*During the annual reproductive cycle in adult mink:* By February Cx46 mRNA levels were highest while Cx50 and Cx43 reached lowest levels in mink tubules fractions (Fig. 2D). As well, the low Cx46 mRNA values recorded by June were accompanied by relatively high Cx50 and
Cx43 mRNA levels (Fig. 2D). Thus, Cx46 mRNA showed profiles opposite to those of Cx50 and Cx43 mRNA not only during postnatal development but during the annual seasonal reproductive cycle as well.

**Cx46 protein expression in normal mink:**

**During development:** The 51- and 68kDa Cx46 immunoreactive band levels both increased with development (Fig. 2E). The increase in 51kDa levels was significant first by 210 days compared to 180 days and again by 240 compared to 210 days thereafter, the 51kDa levels remained elevated by 270 days. When taking into consideration only the 68kDa band, these levels raised significantly by 180 days compared to 150 days and again by 210 days compared to 180 days after that, these levels remained elevated in adulthood (270 days) (Fig. 2E).

**During the annual reproductive cycle in the adult mink:** The 51kDa Cx46 immunoreactive band levels reached low values from August to October. However, these levels peaked by November then remained elevated until April (Fig. 2F). By May, 51kDa levels significantly dropped in comparison to April albeit they transiently increased by July compared to June (Fig. 2F). The profiles of the 68kDa Cx46 immunoreactive band levels were virtually the opposite of the 51kDa Cx46 immuno-reactive band levels. The 68kDa levels were elevated from September to November and again from April to June that is to say, during the time periods when the 51kDa levels were low. By contrast, 68kDa levels diminished from December to March (Fig. 2F) which corresponds to the time periods when the 51kDa levels were elevated. The 68kDa increases were significant from August to September then from March to April while the decreases were
significant by December compared to November and by March compared to February (Fig. 2F). The data indicate that Cx46 is de-phosphorylated during the active spermatogenic phase (Fig. 2F).

**Cx50 protein expression in normal mink**

*During development:* The 51kDa Cx50 immunoreactive band levels were highest by 90 days then steadily decreased by adulthood (Fig. 2G). The decreases of 51kDa levels were significant from 90-120, 180-210 and 210-240 days (Fig. 2G). The levels of the 60kDa Cx50 immunoreactive band remained low during most of development except when they significantly but transiently raised by 240 days (Fig. 2G). Thereafter, 60kDa levels then decreased by 270 while still remaining higher than in 90 days after birth (Fig. 2G).

*During the annual reproductive cycle in the adult mink:* The 51kDa Cx50 levels were augmented from August-November then from May-July by contrast, only traces of 51kDa Cx50 were detected from December to April (Fig. 2H). There were traces of 60kDa Cx50 from August-November then these levels increased from December to April but they decreased again from May to July (Fig. 2H). For the 51kDa Cx50 band, the decrease recorded was significant from November to December while the increase was significant from April to May. For the 60kDa band, the increase was significant from November to December while the decrease was significant from March to April (Fig. 2H). The data indicate the phosphorylation of Cx50 during the active spermatogenic phase (Fig. 2H).

These results show that dephosphorylation of Cx46 and phosphorylation of Cx50 take place during the active spermatogenic phase whereas the opposite takes place during the seasonal testicular regression. The phosphorylation of Cx46 and Cx50 is opposite.
**Cx46 Immunolabelling**

**Mouse:** Controls: No reaction product was detected in Cx46-/- mice testes paraffin sections exposed to Cx46 antibodies (Fig. 3A).

**Development and adulthood:** Cx46 labelling was apparent at the sites of Sertoli cell-to-Sertoli cell and Sertoli cell-to-germ cells contacts by 14 days after birth (Fig. 3C) but not in 7 days old seminiferous tubules (Fig. 3B). The canalisation of a lumen and the colonisation by spermatocytes of most seminiferous tubules were concomitant events that took place typically around 21 days after birth in the mouse (Figs 3D-F). The Sertoli cell intercellular contacts were labelled (Fig. 3F). In addition, the endoplasmic reticulum and Golgi apparatus of spermatocytes exhibited heavy labelling (Figs 3D-F). In addition, Cx46-positive minuscule dots were detected in round spermatids (Figs 3D-F). In the adult mouse, Cx46 labelled contacting Sertoli plasma membranes as well as the endoplasmic reticulum and Golgi apparatus of spermatocytes during the stages of the seminiferous epithelium cycle (Figs 3G-J).

**Mink:** Controls: The controls using either the primary or the secondary antibody alone showed no reaction product (Fig. 3K).

**Development:** The colonisation by spermatocytes of the seminiferous tubules takes place around 180 days after birth in mink. However, in contrast to rodents, the canalisation of a lumen occurs after the completion of spermatogenesis around 240 days after birth in mink (Figs 2A-B). Sertoli cell-Sertoli cell and Sertoli cell-to-spermatogonia or Sertoli cell-to-young spermatocytes contacts established near the base of the epithelium were labelled (Fig. 3L). Minute Cx46-positive halos corresponding to annular junctions were contained within the Sertoli cells trunk (Fig. 3L). Several
lipid droplets were individually surrounded by a Cx46-positive halo (Fig. 3L). An open arrowhead points to a Cx46-positive annular junctions wrapped around a lipid droplet in Figs 3L and 4A. The annular junctions establishing contacts with the lipid droplet surface is clearly apparent in the electron microscopy of both, thin sections (Fig. 4B) and exposed freeze-cleaved membranes (Fig. 4C). Our novel observation here of Cx46-positive Sertoli cell annular junctions enfolding lipid droplets evidences not only the ability of the droplets to communicate with other organelles in Sertoli cells through surface contacts but indicates that Cx46 may be an actor in the lipid droplet-cell junction communication process.

Annual reproductive cycle: In addition to the Cx46 labelling detected in Sertoli cell-to-germ cells contacts (Fig. 3M), some spermatogonia exhibited faint Cx46-positive dots in the Golgi zone adjacent to the nucleus during stage V of the seminiferous epithelium cycle (Figs 3M and 3M'). The perinuclear region, the Golgi zone and the endoplasmic reticulum in leptotene and pachytene spermatocytes were intensely labelled during stage VII (Fig. 3N) and stage VIII (Fig. 3O) that respectively precedes and follows spermiation in the mink. Cx46 labelling was detected in the perinuclear region in spermatocytes and the reaction product was viewed in the basal third of the Sertoli cell-Sertoli cell and in Sertoli cell-germ cell contacts by stage IX (Fig. 3P). The Sertoli cell-to-Sertoli cell contacts and Sertoli cell processes abutting germ cells remaining in the seminiferous tubules were labelled during the seasonal testicular regression (Fig. 3Q).

Cx50 Immunolabelling:
Mouse: Controls: No reaction product was detected when the specificity of Cx50 antibodies was tested in Cx50-/- mice testes paraffin sections (Fig. 5A).

Development and adulthood: Cx50 was virtually undetectable in 7-days-old mouse testis sections (Fig. 5B). However, inter-Sertoli cell contacts set up in the basal third of the seminiferous epithelium at the site of the blood-testis barrier were intensely Cx50-positive in 21 days old (Fig. 5C) as well as in adult mice (Figs 5D-I). In addition, the perinuclear region of spermatocytes contained Cx50-positive material during all stages of the cycle (Figs 5D-I). Minute Cx50-positive dots were also seen in round spermatids (Figs 5E-F). The wall of blood capillaries in the interstitial tissue of the testis was Cx50-positive (Fig. 5I).

Mink: Controls: Controls done with either the primary or the secondary antibody alone showed no reaction product (Fig. 5J).

Development and annual reproductive cycle: A faint and delicate Cx50 labelling was seen along Sertoli cell membranes and in the zones of contacts between them by 60-days after birth (Fig. 5K). By adulthood, Cx50 labelling was apparent in similar locations as the ones described above in the adult mouse namely in Sertoli cells-to-germ cells contacts and inter-Sertoli cell contacts established at the site of the blood-testis barrier established in the basal third of the seminiferous epithelium (Fig. 5L). The distribution of Cx50 remained apparent in Sertoli membranes whether facing Sertoli cells or germ cells remaining in the seminiferous epithelium but was no longer observed at the site of the blood-testis barrier by June (Fig. 5M) and by November (Fig. 5N).

Morphological studies in Cx46-/- and Cx50-/- mouse testes and epididymides
Testis: The seminiferous epithelium contained few germ cells in apoptosis and spermatogenesis appeared normal in both WT (Fig. 6A) and Cx50-/- mice (Fig. 6B). In contrast, Cx46-/- testes sections were characterised by plentiful apoptotic bodies (Figs 6C-E) typically observed amongst spermatocytes of the first and second meiotic divisions (Figs 6C-E) and amongst spermatids (Fig. 6D). Multinucleated giant cells were, however, infrequent (Figs 6C-E). The lumen of some seminiferous tubules was collapsed other contained cellular debris (Fig. 6E).

Quantification of the number of apoptotic germ cells in seminiferous tubule cross sections

A significantly higher number of Apostain-labelled apoptotic cells per seminiferous tubule cross section were recorded in Cx46-/- than in WT and Cx50-/- mice (Fig. 6F). However, the numbers of labelled cells per tubule did not significantly differ between WT and Cx50-/- (Fig. 6F). When measurements of nucleosome release in seminiferous tubule-enriched fractions by ELISA were carried out, significantly higher nucleosome release incidences were found in Cx46-/- than in WT tubule fractions (Fig. 6G) while there were no significantly differences between Cx50-/- and WT (Fig. 6G).

Immunoperoxidase labelling of apoptotic germ cells with monoclonal Ab F7-26 (Apostain)

The adult wild type (WT) (Fig. 6H) and Cx50-/- (Fig. 6I) mouse testis sections hosted occasional Apostain-labelled germ cells in contrast to the Cx46-/- that exhibited multiple aggregations of labelled pachytene and diplotene spermatocytes (Figs 6J-K) and spermatids (Figs 6K-L).
**Epididymis:** The different regions of the epididymides exhibited comparable histological features and showed spermatozoa in similar abundance at the light microscope in WT (Figs 6M-N) and Cx50-/- mice (Fig. 6O). However spermatozoa seem to be in lesser density in Cx46-/- mice epididymides (Figs 6P-R). Apoptotic young germ cells particularly spermatocytes either solitary or clustered were frequently encountered in the lumen of Cx46-/- mice epididymides (Figs 6P-R).

**Cx46-, Cx50- and Cx43-mRNA expression in mink with AIO**

The Cx46 mRNA levels significantly dropped in mink tubule-enriched fractions with AIO by February in comparison to normal mink (Fig. 7A), however, they were significantly increased by March compared to normal mink (Fig. 7A). Cx50 mRNA levels in mink with AIO and normal mink were not significantly different (Fig. 7B). The Cx43 mRNA levels significantly increased in mink with AIO by February. The levels fell significantly by March in comparison to normal values (Fig. 7C).

**Cx46 protein expression in mink with AIO**

The 51-kDa Cx46 immunoreactive band levels significantly decreased in contrast to the 68-kDa band levels that augmented in mink with AIO by February compared to normal mink (Figs 7D-E) suggesting an increase in phosphorylated Cx46. As well, by March, the 51kDa Cx46 levels significantly decreased and the 68 kDa Cx46 levels augmented in orchitic tubules compared to normal suggesting again an increase in phosphorylation (Figs 7D-E).

**Cx50 protein expression in mink with AIO**
The levels of the 51-kDa Cx50 immunoreactive bands significantly increased in tubules of mink with AIO compared to normal by February (Figs 7D, 7F). The 60kDa band levels fell significantly by March in mink with AIO compared to normal values (Figs 7D, 7F). Profiles of Cx46 and Cx50 protein levels and phosphorylation status were opposite in mink with AIO (Figs 7D, 7F).

**Cx43 protein expression in mink with AIO**

The levels of Cx43 significantly increased by February but dropped by March in mink with AIO compared to the normal (Figs 7D, 7G) thus emulating the Cx50 profiles (Figs 7D, 7F).

**Morphological studies mink testes with AIO**

Spontaneous AIO was characterized by germ cells exfoliation (Fig. 7H), multinucleate giant cells formation (Fig. 7K), vacuolization of Sertoli cells (Figs 7I-L, N) and arrest of spermatogenesis at different stages of germinal cell development (Figs 7I-L, N) and (75; 82). The release of cohorts of germ cells gave a vacuolated appearance to seminiferous epithelium (Figs 7I-L, N). The lumen was collapsed in some tubules (Figs 7I-J), others were infiltrated by cells from the immune system. Only Sertoli cells and spermatogonia remained in severely damaged tubules (Figs 7I, J, N).

**Cx46 immunolabelling**: Labelling was observed in minute vesicles in Sertoli cells (Fig. 7H), the cell membrane of large vacuoles and facing Sertoli cell membranes (Figs 7H-J).
Cx50 immunolabelling: Sertoli cell membranes facing early-stage germ cells were labelled (Fig 7L). In addition, the perinuclear region of remaining spermatocytes was Cx50-positive (Fig. 7L). As well, the wall of blood vessels (Fig. 7M) was labelled. However, severely damaged tubules exhibited insignificant labelling (Fig.7N).
DISCUSSION

Cx46 and Cx50 in the seminiferous epithelium

Cx46 has been studied in osteoblastic cells (41), astrocytes (24), alveolar epithelial cells (1), bone (90), human breast tumour (7), heart (20) and lens (5; 32; 33). Cx50 has also been studied in the lens (31; 103). However, this is the first report on Cx46 and Cx50 in the mouse and mink seminiferous epithelium.

Cx46 and Cx50 expression and phosphorylation

Cx46: Our finding of a 51kDa and a 68kDa phosphorylated Cx46 immuno-reactive band in mouse and mink seminiferous tubules fractions agrees with the report of bands of similar molecular mass in other rodent tissues and cultured cells (19; 41). As well, our observation of a phosphorylated form of Cx46 in tubules fractions is consistent with the report of the involvement of casein kinase and protein-kinase C (PKC) (88) in the phosphorylation of Cx46 in threonine and serine residues in lens (9; 100).

Cx50: Our observation of a 51kDa Cx50 immuno-reactive band in mouse and mink lens and tubules fractions agrees with the report of a similar band in murine lens (93). Moreover, our finding of a phosphorylated 60kDa Cx50 form is consistent with the report of the phosphorylation of Cx50 on serine 395 and threonine residues by protein kinase A (PKA), PKC- and external regulated protein kinase/ERK in the lens (49; 51; 93).

Evidence for an interactive regulation of Cx46, Cx50 and Cx43 in the seminiferous epithelium
The 51kDa Cx46 level profiles were opposite to those of the 51kDa Cx50 and Cx43 in mouse developing tubule fractions. As well, the 51kDa Cx46 and 51kDa Cx50 profiles were opposite during development in mink. Moreover, we found that the phosphorylation of Cx46 and Cx50 is also opposite during mouse development as well as during the normal mink annual seasonal reproduction cycle. The change in the phosphorylation status of Cx46 and Cx50 in addition to being opposite takes place from November to April that is to say during the active spermatogenic phase. This advocates for a timely and interdependent regulation of not only the expression but also of the phosphorylation of Cx43, Cx50 and Cx46 in the seminiferous epithelium. In mouse, deleting Cx50 results in a decrease in phosphorylated 68kDa Cx46 while deletion of Cx46 causes an increase in 51kDa Cx50 accompanied by a decrease in the phosphorylated 60kDa Cx50. Moreover, deletion of Cx50 is accompanied by a dramatic increase in PCx43 (phosphorylated in serine 368). Together, these findings evidence a complementary expression and phosphorylation of Cx46 and Cx50 and show that the expression and phosphorylation of one connexin influences the expression and phosphorylation of other connexins in the seminiferous epithelium. Our observations are in line with the report of an up-regulation of Cx46 in regions of the lens where Cx43 was down-regulated (60) and with the inverse relation in Cx43 and Cx46 protein amounts reported in Y79b retinoblastoma cells xenografts in human (15).

*Evidence for an influence of Cx46, Cx50 and Cx43 on other junction constituents in the Sertoli cell junctional complexes.*

Our observation of Cx46 interacting with other cell junction components besides or in addition to Cx50 and Cx43 reflects the reciprocal influence of individual cell junction constituents
on each other within Sertoli cell junctional complexes. Cx46 deficiency causes a total Cx43 levels increase and an occludin and N-Cadherin decrease. Cx50 deficiency results in increased total Cx43 and Claudin11 levels and dropping ZO-1 levels in mice. Thus, when tight junctions and adherens junctions proteins are affected by Cx46 deletion they are not affected by the deletion of Cx50 and vice versa. Our finding of an interaction of Cx46 and Cx 50 with ZO-1 in the seminiferous epithelium agrees with the report of a Cx46 and Cx50-ZO-1 interaction in other cell systems (65). As well, our observation is also in agreement with an earlier report of a Cx43 increase accompanied by a decrease in N-cadherin, occludin and claudin-11 protein levels in the seminiferous tubules during development and the annual reproductive cycle in mink (74). In whole mouse testis extracts, the conditional invalidation of Cx43 in mouse Sertoli cells was found to increase N-cadherin, Ξ-catenin, occludin but to decrease ZO-1 protein levels (18). Blocking gap junction-mediated cell coupling with gap junction inhibitors and siRNA to decrease Cx43 levels causes similar effects in a Sertoli cell line (18). The interdependence amongst individual constituents within the Sertoli cell junctional complex entails that modifications targeted to a single constituent alter the expression and phosphorylation state of other junction constituents and likely the function these constituents regulate.

The timely transition of Cx43 to Cx46 and Cx50 expression and phosphorylation

The regulation of the expression and phosphorylation of Cx46, Cx50 and Cx43 is timely with the collection of particular generation germ cells in the seminiferous epithelium. Our finding here that total Cx43 protein levels decrease with adulthood in the tubules confirms earlier observations in mouse (99). Moreover, we have documented a peak in tubular Cx43 levels
concurrent with the onset of meiosis and the colonisation of the tubules by spermatocytes (74; 75) an observation consistent with the localization of Cx43 mRNA to primary spermatocytes (86). This study localises Cx50 and Cx46 in the perinuclear region of spermatocytes and found that the Cx43 levels elevated by the beginning of mouse and mink development, during cellular growth and proliferation dropped during maturation in adulthood. The profile of Cx50 expression emulates that of Cx43. By contrast, the low 51- and 68kDa Cx46 expression early in development augments with adulthood. In the mink, we found that the phosphorylation status of Cx46 and Cx50 is opposite during the active spermatogenic phase. Together, these observations are indicative of a stage-specific transition of Cx43 and Cx50 to Cx46 expression taking place during the switch from the mitotic to the meiotic division, the colonization of the tubules by spermatocytes and their translocation into the lumenal compartment of the seminiferous epithelium.

The regional distribution of Cx46, Cx50 and Cx43 in the seminiferous epithelium

We have earlier reported that PCx43 phosphorylated in serine 368 is localised to gap junctions established at the site of the blood-testis barrier (99). Our observation here that phosphorylated Cx50 increased during the active spermatogenic phase and that during this period Cx50 has a distribution reminiscent of that of PCx43 is indicative of an implication of Cx43 and Cx50 phosphorylation in the dynamics of this barrier by contrast to Cx46 that exhibits a different distribution within the seminiferous epithelium. During the mink active spermatogenic phase, Cx50 became phosphorylated (Fig. 2H) and localized to the site of the blood-testis barrier (Fig. 5L) by contrast to Cx46 that was dephosphorylated (Fig. 2F) and associated with annular junctions during the same period suggesting the involvement of Cx46 and Cx50
phosphorylation/dephosphorylation in the dynamics of this barrier. Cx46-positive annular junctions establishing contacts with the lipid droplet surface were found indicating the participation of Cx46 in the touch and degradation process. We have reported that the blood testis barrier is competent in blocking entry of blood borne substances from December to March in this species (Fig. 2B) (68; 69). Phosphorylation of Cx43 has been shown to influence junction permeability by regulating the connexin degradation process (30). Phosphorylation of Cx50 by PKC-(uncouples cortical fibre cells in the lens, and reduces Cx50-mediated communication in contrast to phosphorylation by PKA that augments it (51). Thus, transitory changes in the phosphorylation status of Cx50 could in theory momentarily impede Cx50-mediated junction communication established at the site of the blood-testis barrier while uncoupling germ cells and Sertoli cells in accordance with the physiological requirements of the spermatogenic activity.

In addition, we localised Cx50 and Cx46 to the perinuclear region of spermatocytes. This study also reveals a transition of Cx43 to Cx46 expression during spermatogenesis. Significantly, this transition to Cx46 expression coincides with a drop in the oxygen levels available to spermatocytes in transit from the normo-oxygenated basal compartment into the hypoxic lumenal compartment of the seminiferous epithelium. Whether the up regulation of Cx46 in late-stage germ cells is causally related to the drop in oxygen supply inflicted to these cells by the impediment of a direct access to circulation imposed by the blood-testis barrier remains to be established. This notwithstanding, this view is compatible with the report that hypoxia upregulated Cx46 in epithelial cells and that exogenous Cx46 prolonged mouse N2A cells outliving from hypoxia-induced cell death (7). Here, Cx46 deficient mice show significant increases in apoptosis levels measured by ELISA as well as in the number of Apostain-positive spermatocytes and spermatids.
indicating that Cx46 gap junction-mediated exchanges are life-sustaining for these late-stage germ cells. In addition, our finding of Cx46 in the perinuclear region of selected germ cells raises the possibility that Cx46 could serve as a marker for an intermediate germ cell phenotype, the meiosis.

The spermatocytes may be unique in their capacity to transiently house besides or in addition to Cx43 (86), a pool of Cx46 and Cx50. Because the spermatocyte stage extends over several days, opportunities for the Cx46 “retained” in the perinuclear compartment to interact with other cell junction proteins like Cx50 and Cx43 available within the cell or neighbouring cells are favoured. Whether this inter-relation is carried out awaiting Cx46 oligomerization is unknown but the view is not incompatible with the report of connexin oligomerization being required for the transport from an intracellular compartment to the cell surface (42; 64). Our observation of Cx46 in vesicles as well as in junctional plaques at the surface of Sertoli cells, argues favourably for Cx46 at times accumulating in vesicles at other times being transported to and retained at the plasma membrane in response to various stimuli. Together these observations raise the possibility of a transit of Cx46 between the perinuclear region and the cell surface in spermatocytes before disposal in Sertoli cells. Cx46 has been shown to be “retained” in a trans-Golgi compartment in several cell systems including cultured osteoblastic cells (41), lens cells (6) and canine bone tissue (90).

In addition, our observation evidences the biological significance of Cx46 not only on spermatocytes but on Sertoli cells physiology as well. The infrequency of apoptotic figures we typically observed in wild type normal mouse testis sections despite the documentation of seventy five percent of the potential number of spermatozoa being lost through apoptosis-mediated germ cell death (35; 39) is proof of the efficient removal and disposal of cellular debris by Sertoli cells. However, our observation of an overload of apoptotic germ cells in Cx46 deficient mice indicates
that imbalances in Cx46 gap junction-mediated exchanges result in the phagocytic clearance ability of Sertoli cells being exceeded or in an alteration of the Sertoli cell phagocytic function. Here, the targeted deletion of Cx46 increases nucleosome releases measured by ELISA and causes an overload of dying Apotain-positive cells in situ that is to say, of damaged that are non-engulfed germ cells by Sertoli cells. By contrast, AIO was characterised by an overload of apoptotic and giant cells without modification of apoptosis levels in the early stages of the disease (82).

**Cx46 and Cx43 and blood-testis barrier**

Our observation that Cx46 and Cx50 mRNA and protein are expressed and localised in seminiferous tubules is novel. Most tissue cells host more than a single isotype cloned connexins (84). Cells expressing several species of connexins have the ability to form mixed gap junction channels (13; 102; 104). Cx46 (the \( \forall \)3 connexin) differs from Cx50 (the \( \forall \)8 connexin) in that it can exceptionally form functional heteromeric gap junctions with either Cx43, an \( \forall \)-connexin, or Cx32 a \( \exists \)-connexin in Xenopus oocytes (104) and cultured primary alveolar epithelial cells (1). The molecular diversity of hemichannels results in heterotypic gap junction channels that influence a wide variety of cellular functions like growth and differentiation (16; 40). Moreover, the identity of connexins available to cells dictates the mode of gap communication through ionic or biochemical coupling.

The seminiferous epithelium is singular in that many germ cells undergoing distinct life stages are in contact with a unique Sertoli cell. Yet, the physiological requirements imposed by individual life stages differ from mitosis to meiosis. On the one hand, gap junction-mediated communication helps synchronise interconnected Sertoli cells activities over some distance in
seminiferous tubules. On the other hand, the gap junction-mediated Sertoli cell-germ cell communication favours coordination of germ cell and Sertoli cell activities. This coordination is vital because it helps germinal cells to carry out functions unresolvable to themselves alone timely with their individual life stages (73-75). In this context, Cx46 endogenously expressed in the spermatocytes that are involved in the translocation from one side to the other of the blood-testis barrier may take on a physiological role. Connexins can be mechanosensitive, the response being connexin-specific (8). The gap junction channels open to allow exchanges between cell interiors usually only after two hemichannels dock. A singularity of Cx46 and Cx50 resides in that these connexins can provide both, gap junction channels as well as non-junctional conductances in contrast to other connexins that typically form closed hemichannels in non-junctional cellular membranes (29). In *Xenopus laevis* oocytes, Cx46 (67) and Cx50 (107) both form open hemichannels when expressed exogenously. Hemichannels and complete gap junction channels share the same permeability and gating characteristics albeit open hemichannels can endanger the cell because they are non-selective. For instance, *Xenopus* oocytes expressing Cx46 or Cx50 exogenously will survive only if the hemichannels are maintained closed by high extracellular Ca$^{2+}$ concentration (28). The increased number of apoptotic number of spermatocytes we measured in Cx46/- mice suggest that hemichannels are not maintained closed following this genetic deletion. In contrast to Cx46 and Cx50, ubiquitous Cx43 forms open hemichannels only when challenged with nonphysiological extracellular Ca$^{2+}$ concentration (48).

*Cx43, 46 and Cx50 in lipid rafts*
Our finding of Cx43 in rafts in seminiferous tubules fractions agrees with the report of Cx43 in two cholesterol-binding integral membrane proteins of a subpopulation of lipid raft domains: caveolin-1 (45; 50; 91), and caveolin-2 (45) in other cell systems. As well, our observation of Cx46 and Cx50 in rafts in seminiferous tubule fractions is in line with the localisation of these two connexins in lens caveolae (49) but differs from the localisation of only Cx46 in lipid rafts of the lens (91). The high concentration of sphingolipids and cholesterol of the lipid rafts favours the accumulation of specific proteins in these microdomains. Cx46- and Cx50-mediated gap junctions are enriched in cholesterol and sphingomyelin (4; 34) suggesting that connexin channels can segregate into lipid rafts. The functional significance of rafts in the regulation of membrane channels is based on their contribution to intracellular signaling interactions (21; 94) and their ability to selectively include or exclude proteins from individual membrane domains (83). The sorting controls the delivery, assembly and removal of individual proteins from selected microdomains (44; 87; 92). Each ~8 nm gap junction intramembranous particle visualised on the P-fracture face of the plasma membrane (Fig. 4C) corresponds to one connexon or hemichannel and the pairing of two of these units results in a channel. The filipin cytochemistry has allowed us to establish that gap junction formation/dismantling occurs in lipid-rich microdomains while junction maturation occurs in cholesterol-poor domains (74; 76; 77; 81). This observation indicates that individual connexin-mediated channels are sorted in different microdomains according to connexin species or pairing characteristics through differences in lipid/protein ratios in Sertoli cell membranes.

AIO lipid raft and Cx43 and Cx46.
The tumour necrosis factor-∀ TNF∀ mRNA and protein were reported in round spermatids (22) while interleukin-1∀ (IL-1∀) and interleukin-6 (IL-6) are secreted by Leydig and Sertoli cells (66). The interferon gamma (IFN() mRNA and protein were recorded in phytohaemagglutinin-stimulated early spermatids but not in peritubular cells, Sertoli cells, or pachytene spermatocytes (23). Spontaneous AIO causes the release of TNF-∀, IL-6 (82) and IFN( (unpublished observations) associated with aberrations in the gene transcription and post-translational phosphorylation of Cx43 (75). TNF∀ and interleukin-1∃ (IL-∃) have been shown to modify Cx43 turnover in folliculostellate cells (30; 59). The release of proinflammatory cytokines triggered by AIO may alter the recruitment and sorting of Cx46, Cx50 and Cx43 channels into areas of the Sertoli cell plasma membrane possessing a rigidity related to that of “rafts” resulting in modifications in the behaviour, function and future of these gap junctions. This hypothesis will require comparing the collection of Cx46, Cx50 and Cx43 in the lipid rafts of cells in orchitic tubules with that in normal. This notwithstanding, the view is compatible with the following two reports in different cell systems. In MDCK cells, TNF∀-triggered translocation of TNFR1 and c-Src into lipid rafts stimulates an intracellular pathway that deregulates gap junction intercellular communication (25). Similarly, the release of IFN( by enterocytes during necrotising enterocolitis displaces Cx43 from lipid rafts impairing Cx43 in these cells and inhibiting their migration (46).

\textit{Cx46 localizes in junctions around lipid droplets:}

Our earlier observation of Sertoli cell lipid droplets establishing contacts with cholesterol-positive cell junctions, cisternae of endoplasmic reticulum and junctional segments blebbing from the cell surface has evidenced the ability of the droplets to communicate with other intracellular
organelles through stable or transient surface contacts in the seminiferous epithelium (74). Lipid droplets-cell junction contacts have been documented in other cell systems (57; 106). Our finding here of Cx46-positive annular junctions establishing contacts with the lipid droplet surface in Sertoli cells is novel and is indicative of the participation of Cx46 in the process. Proteomic studies have identified several proteins on the lipid droplets surface in macrophages (36) and uncovered the traffic of membrane proteins and the implication of intracellular membranes and lipid droplet contacts (12; 52; 53; 56).
PERSPECTIVES AND SIGNIFICANCE

The establishment of a Sertoli cell occluding zonule barrier in the basal third of the seminiferous epithelium creates a basal cellular compartment superposed by a lumenal compartment. The early-stage germ cells hosted in the basal compartment benefit from a direct access to blood-borne substances while the late-stage germ cells dwelling the lumenal compartment do not. The feature of a lack of a direct access to the blood supply is shared by the late-stage male germ cells and the fibre cells of the naturally avascular lens of the eye where Cx46 and Cx50 have been initially scrutinised. However, this is the first report of Cx46 and Cx50 in testis. The increase recorded here in apoptosis levels and in the number of apoptotic Apostain-positive spermatocytes and spermatids in \( \text{Cx46}^{-/-} \) mice argues in favour of life-sustaining Cx46 gap junction-mediated exchanges in the late-stage germ cells that are secluded from the blood by the barrier. Moreover, our findings of a down-regulation of Cx46 concomitant with an up-regulation of PCx43 in \( \text{Cx50}^{-/-} \) mice and of an up-regulation of Cx50 and Cx43 in \( \text{Cx46}^{-/-} \) mice is evidence that the regulation of Cx46 is complementary not synchronous with Cx50 and Cx43 in the seminiferous epithelium. Furthermore, phosphorylation of Cx46 and Cx50 is opposite or complementary in the seminiferous epithelium. As well, the increase in PCx43 levels recorded in \( \text{Cx50}^{-/-} \) mouse tubule fractions is reflective of the individual cell junction constituents influence on the phosphorylation state, and likely on the function regulated by other junction proteins within the Sertoli cell junctional complexes. The data advocate in favour of an interdependent regulation of Cx43, Cx50 and Cx46 expression and phosphorylation in the seminiferous epithelium. In addition, the data show that when Cx46 affects the tight junction and adhering junction proteins, Cx50 has no effect and vice versa indicating that deleting \( \text{Cx46} \) or \( \text{Cx50} \) impacts the behaviour of other constituents within the
Sertoli cell junctional complex. During the mink active spermatogenic phase, Cx50 became phosphorylated and localized to the site of the blood-testis barrier by contrast to Cx46 that was dephosphorylated and associated with annular junctions suggesting involvement of Cx46 and Cx50 phosphorylation/dephosphorylation in the dynamic of this barrier. Our novel detection of Cx46-positive annular junctions establishing contacts with the lipid droplet surface is indicative of the participation of Cx46 in the touch and degradation process. Our finding of Cx46 Cx50 and Cx43 in lipid rafts may reflect the sorting out of individual connexin-mediated channels in Sertoli cell membrane microdomains according to connexin species or pairing characteristics through differences in lipid/protein ratios. In controlling the traffic of regulatory molecules responsible for these functions, the molecular diversity of connexins regulates cell and tissue growth, cell differentiation and death during spermatogenesis. The diversity in functions of signalling, permeability, voltage gating and interaction of channels with connexins in bordering cells is echoed by the molecular diversity of constitutive connexins. This study paves the way to invaluable opportunities to explore unsuspected consequences of altering the coding of Cx46 and Cx50 not only on the remaining junctional constituents expression and post-translational transformation but more importantly on their individual functions within the Sertoli cell junctional complex that forms the anatomical basis of the blood-testis barrier.
ACKNOWLEDGEMENTS

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FIGURE LEGEND

Figure 1 (A) The samples were subjected to RT-PCR using primers specific for mouse Cx46, Cx50 and/or Cx43 gene. The Cx46, Cx50 and Cx43 mRNA levels in normal mouse seminiferous tubule-enriched fractions during mouse postnatal development are shown. The values are the mean ± SEM of three independent experiments for each age group expressed in arbitrary units. Cx46 mRNA levels significantly increased from 14d to 21d (†P<0.03 21d vs 14d) then, raised further by >60d in contrast to Cx50 mRNA levels that significantly decreased from 14d to 21 d (@P<0.02 21d vs 14d) then remained low by >60 days. The Cx43 mRNA levels steadily decreased throughout development reaching lowest values by >60days (*P<0.05 >60d vs 21d). (B) When exposed to the polyclonal Alpha Diagnostic Cx46 antibody, a single intense ~51kDa band was detected in adult mouse (mo) lens used as a positive control. An additional ~68kDa band was however recognised in both mouse (mo) and mink (mi) seminiferous tubule-enriched fractions (STf). The Cx50 antibody detected a 51kDa immunoreactive band accompanied by a ~60kDa in the adult mouse (mo) lens used as a positive control. The 51-and ~60kDa Cx50-immunoreactive bands were detected in mouse (mo STf) and mink (mi STf) tubule-enriched fractions. October (Oct.) February (Feb.). (C) Phosphorylation status studies of Cx46 and Cx50: Normal adult mink tubule-enriched fractions in February (Feb.) were incubated in PBS either alone or containing alkaline phosphatase (Ph) in the absence (-) or presence (+) of phosphatase inhibitor (I). Following treatment, total protein aliquots from each sample were subjected to SDS-PAGE followed by Western blotting with either Cx46 or Cx50 antibodies. A ~68- and 51kDa bands were detected by Cx46 antibodies. Both bands diminished after alkaline phosphatase treatment revealing the
presence of Cx46 phosphorylated forms. The strong 60kDa band detected by Cx50 antibodies was decreased following alkaline phosphatase treatment but the 51kDa band increased suggesting the presence of phosphorylated forms in the 60kDa- Cx50 immunoreactive band. (D) Experiments carried out using detergents to isolate the DIG fractions from adult mink tubule-enriched fractions obtained in February: Caveolin-1 (Cav1) and flotillin-1 (Flot1) served to identify the DIG fraction by Western blotting. The gap junction proteins Cx46, Cx50, Cx43 as well as the tight junction proteins occludin (Occl) and claudin 11 (Cld11) were recovered in the DIG fraction. (E, F, G) Representative Western blots analyses and quantification of individual levels of (E) the 51- and ~68 kDa Cx46 immunoreactive bands, (F) the 51- and ~60 kDa Cx50 immunoreactive bands and (G) total Cx43 (Cx43) measured in mouse tubule-enriched fractions during development are shown. The values are the mean ∀ SEM of three independent experiments expressed in arbitrary units. (E) The changes in Cx46 protein levels were significant as follows: 51kDa: +P<0.01 35d vs 28d and †P<0.03 42d vs 35d; 68kDa: +P<0.01 21d vs 14d and >60d vs 21d. (F) The following changes in Cx50 levels were significant: 51kDa: +P<0.01 35d vs 14d and >60d vs 42d; 60kDa: *P<0.05 21d vs 14d and 42d vs 21d, +P<0.01 60d vs 42d. (G) The following changes in Cx43 protein levels were significant (+P<0.01 21d vs 14d, *P<0.05 28d vs 21d and #P<0.0001 >60d vs 14d). H-I-J are compensatory studies carried out in Cx46−/−, WT and Cx50−/− mice tubule-enriched fractions. The values shown are the mean ∀ SEM of three independent experiments and are expressed in arbitrary units. (H’ and H”) Representative Western blots accompanied by histograms of the quantification of Cx46 and Cx50 levels in each animal group are presented. As expected, the intensity of the Cx46- (H’) and of the Cx50 (H”) immunoreactive band levels were reduced to insignificant traces in Cx46−/− (51kDa band: +P<0.01 Cx46−/− vs WT and 68kDa band: ++P<0.001
**Cx46-/− vs WT** and **Cx50-/− (51kDa band: **P<0.005 Cx50-/− vs WT; 60kDa band: &P<0.0005 Cx50-/− vs WT) mice respectively. (H') The 68kDa Cx46 band levels decreased significantly (@P<0.02 Cx50-/− vs WT) in Cx50-/− mice. (H") The 51kDa Cx50-immunoreactive band levels significantly increased (#P<0.0001 Cx46-/− vs WT) while 60kDa levels were reduced (+P<0.001 Cx46-/− vs WT) in the Cx46-/− mice. Representative Western blots of (I) PCx43, Cx43 and (J) Occludin, Claudin 11, ZO-1 and N-Cadherin levels accompanied by histograms of the quantification of the levels measured for each junction protein in WT, Cx46-/− and Cx50-/− mice tubule-enriched fractions. The following changes in the levels of each junction protein were significant: ††P<0.003 P-Cx43 Cx50-/− vs WT; **P<0.005 Cx43 Cx46-/− vs WT; *P<0.05 Cx43 Cx50-/− vs WT;*P<0.05 Occludin Cx46-/− vs WT; *P<0.05 Claudin 11 Cx50-/− vs WT; *P<0.05 ZO1 Cx50-/− vs WT;*P<0.05 N-Cadherin Cx46-/− vs WT.

Figure 2. Variations in the Cx46, Cx50 and Cx43 mRNA and protein levels in mink seminiferous tubule-enriched fractions during postnatal development and the annual reproductive cycle. A calendar of the germ cell population recorded during (A) the postnatal development and (B) the annual seasonal reproductive cycle in mink seminiferous tubule cross sections is provided. The mink breed in the second and third weeks of March. The pups are born in April-May. Each month is represented by a vertical column in the two diagrams. The columns either span (A) from birth to adulthood by 270 days after birth or (B) cover the twelve-month seasonal reproductive cycle of the adult mink. The shading refers to time periods when the blood-testis barrier is permeable to vascularly infused permeability tracers. Conversely, the months when the barrier is competent in blocking tracers are not shaded. The inner circle apposed atop the diagram indicates the presence
of a lumen. (G: Gonocytes; Pre-A: pre-type A spermatogonia; A₀d: A₀ spermatogonia dividing; A:
type A spermatogonia; PL: pre-leptotene spermatocyte; P: pachytene spermatocyte; 7: step 7
spermatid; 19: step 19 spermatid). After a 90 days neonatal period, puberty encompasses the
colonisation of the tubules by type A spermatogonia until the reaching of the first spermatozoa in
the epididymis some 250 days after birth. This is followed first by an “active” then, by an
“inactive” phase of the annual reproductive cycle in the adult mink. A reduction in mitotic and
meiotic activities yields spermatids in reduced abundance. By August (AUG), the division of
spermatogonial stem cells (A₀d) marks the onset of the active phase of the annual seasonal
reproductive cycle (after Pelletier 1986 and modified by Pelletier et al 2009) (68; 82). The
individual Cx46, Cx50 and Cx43 mRNA levels measured by RT-PCR in tubule-enriched fractions
are plotted in arbitrary units during (C) development and (D) the annual seasonal reproductive
cycle in the adult mink. The values are the mean ± SEM of three independent experiments and are
normalised to 120 days values in C and to August values in D. (C) The Cx46 mRNA levels
significantly increased (*P<0.05 270d vs 120d) however, Cx50 decreased (#P<0.0001 270d vs
120d) by adulthood. Similarly, Cx43 significantly fell (Cx43 ##P<0.00001 180d vs 120d) by 180
days after birth then remained low in the adult. (D) During the seasonal reproductive cycle, Cx46
mRNA levels were highest in February (+P<0.001 Feb. vs Aug.) but then significantly decreased
by June (**P<0.005 June vs Feb.). By contrast, Cx50 mRNA levels were lowest in February
(**P<0.005 Feb. vs Aug.) before significantly increasing by June (*P<0.05 June vs Feb.). The
Cx43 levels were significantly lowered by February (&P<0.0005 Feb. vs Aug.) but significantly
increased by June (**P<0.005 June vs Feb.). (E, F) Representative Western blots with histograms
showing the monthly changes in Cx46 protein levels in tubule-enriched fractions during (E)
development and (F) the annual reproductive cycle in mink. Quantification of the levels of the 51- and 68kDa Cx46-immunoreactive bands are provided. The values are the mean ∀ SEM of three independent experiments expressed in arbitrary units and normalized to the 51kDa band in 90 days old mink for studies on development and normalised to August during the annual reproductive cycle. The changes in Cx46 protein levels were significant during (E) development (51kDa band: *P<0.05 210d vs 180d, **P<0.005 240d vs 210d) and (68kDa band: *P<0.05 180d vs 150d, **P<0.005 210d vs 180d) and (F) the seasonal reproductive cycle (51kDa band: ++P<0.001 Nov. vs Sept and May vs April, *P<0.05 July vs June) (68kDa: +P<0.01 Sept vs Aug. and April vs March, *P<0.05 Dec. vs Nov., March vs Feb. and July vs June). (G, H) Representative Western blots of Cx50 in mink tubule-enriched fractions during (G) development and (H) the annual reproductive cycle. The quantification of the 51- and 60kDa Cx50 immunoreactive band levels are plotted monthly. The values are the mean ∀ SEM of three independent experiments expressed in arbitrary units and normalized to the 51kDa band in the 90 days old during development and to August during the annual reproductive cycle. (G, H) The following changes in Cx50 levels were significant during development (51kDa band: ++P<0.001 120d vs 90d, *P< 0.05 210d vs 180d, +P<0.01 240d vs 210d) and (60kDa band: ++P<0.001 240d vs 210d, *P<0.05 270d vs 240d) and the annual reproductive cycle (51kDa band: @P<0.02 Dec. vs Nov and May vs April) and (60kDa band: *P<0.05 Dec. vs Nov. and April vs March).

Figure 3. Immunoperoxidase labelling with anti-Cx46 in (A-J) mice and (K-Q) mink. (A) No reaction product is detected with Cx46 antibodies used on Cx46-/- mice testis sections. (B) Cx46 labelling was virtually undetectable in this seven-day old testis section. (C) Sertoli cell plasma
membranes whether facing each other near the centre of this developing tubule or engaged in Sertoli cell-to-Sertoli cell and Sertoli cell-to-germ cells contacts near the basal third are heavily labelled (open arrowheads) by 14 days. (D-F) The colonisation by spermatocytes of the seminiferous tubules and canalisation of a lumen are seen in this 21 days old mouse testis section. The open arrowheads point to labelling at Sertoli cell (S) plasma membranes (F) and (E) intercellular contacts. The endoplasmic reticulum and Golgi apparatus of (E) zygotene (Z) and (D, F) pachytene (P) spermatocytes are heavily labelled. (G-J) As well, Sertoli plasma membranes (open arrowhead in G) and the endoplasmic reticulum and Golgi apparatus of pachytene (P), zygotene (Z) and diplotene (Di) spermatocytes are labelled in adult mice. The stages of the cycle of seminiferous epithelium appear at the top of the micrographs. Spermatogonia (g). (K) No immunostaining is detected in Cx46 controls done on a normal adult mink testis section obtained in February when using the primary or secondary antibody alone. (L) Cx46 immunolabelling is shown in a 210 day-old and (E-I) adult mink testes in (M, M', N, O, P) February and (Q) August. (L) Labelling (open arrowhead) is identified in variously sized vacuoles scattered within the trunk of Sertoli cells (S) as well as in Sertoli cell contacts with the germ cells (spermatogonia (g); pachytene spermatocytes (P)). Lipid droplets (l) near the base of Sertoli cells are surrounded by an intense Cx46-positive halo (open arrowhead). (M, M’, N, O, P) In February, Cx46 labelling is seen (open arrowheads) in regions of Sertoli cells and in their contacts between themselves and with spermatogonia (g), leptotene (L) and pachytene (P) spermatocytes) during the different stages of the mink seminiferous cycle appearing in roman numerals atop of the figures. The inset in M’ shows a higher magnification of Cx46 labelling in the Golgi region of the spermatogonium identified g# in M. The endoplasmic reticulum and the Golgi zone of the leptotene (L) and
pachytene (P) spermatocytes are labelled. Cx46 labelling decreased in spermatocytes (P) from stage VIII to (O) stage IX. (Q) During the seasonal testicular regression, Cx46 labels (open triangles) thin Sertoli cells (S) processes some of which being in contact with spermatogonia (s) and pachytene spermatocytes (P) remaining in seminiferous tubules by August. Lipofuscin pigments are identified (asterisks) in regressed Sertoli cells (S). g: spermatogonia. A-M; O-Q: X 860; M’: X 980.

Figure 4. (A) This is a higher magnification of a portion of the field shown in Figure 3L in which the attention is drawn here to Cx46 labelling detected (open arrowheads) not only in Sertoli cell-to-germ cell junctions but as well in annular junctions found either wrapped around a lipid droplet (l) or scattered within the trunk (open arrowhead) of the Sertoli cell (S). Electron microscopy of thin section of an annular junction corresponding to the one shown in A is shown in B establishing contacts with the surface of a lipid droplet. A corresponding image is shown in C in electron microscopy of freeze-fracture. In this micrograph, an impressive circular array of Sertoli cell annular gap junctions (open arrowheads) intercalated amongst strands of tight junctional particles (arrows) is seen surrounding a cluster of filipin-cholesterol complexes (closed arrowheads). A: X1,000; B: X1000,000; C: X116,000.

Figure 5. Immunoperoxidase labelling with anti-Cx50 in (A-I) mice and (J-N) mink testis sections. (A) No reaction product was detected when Cx50 antibodies were used on Cx50/-/- mice testis sections. (B) No reaction product was detectable in seven days old mice testis sections. (C) This 21-days-old mouse testis section shows Cx50 labelling (open arrowhead) in the cell contacts
settled at the site of the blood-testis barrier. (D-H) At this site, Cx50 labelling is seen in cell contacts established above spermatogonia (g) and young spermatocytes in the basal third of the seminiferous epithelium during the stages cycle in adult mice. (D-J) In addition, zygotene (Z) and pachytene (P) spermatocytes show intense Cx50 labelling. Di: diplotene spermatocytes. (I) The wall of the capillary is labelled. (J) Cx50 control done using either the first or the second antibody in adult mink testis sections obtained in February shows no immunostaining. (K) The Sertoli cell (S) membranes show delicate labelling (open arrowheads) in a 60 day (d)-old mink. G: undifferentiated spermatogonia. (L) In February, the distribution of Cx50 (open arrowheads) above spermatogonia (g) and young spermatocytes in the tubules closely coincides with that of junctional complexes established at the site of the blood-testis barrier in mink. (M) The lumen of the seminiferous tubule is collapsed in June during testicular regression and contacting Sertoli cell plasma membranes are labelled however, the distribution of Cx50 labelling no longer coincides with that of the junctional complexes at the site of the blood-testis barrier. Contacts between Sertoli cells and spermatogonia (g) pachytene spermatocytes (P) are labelled in November. As well, the Golgi zone of the spermatogonium identified g*. std: spermatid contains minuscule Cx50-positive dots. (A-N): X 860.

Figure 6. A-E Bouin’s-fixed Periodic Acid Shiff (PAS)-stained paraffin testis sections from (A) normal >60 days old wild type (WT), (B) Cx50-/− and (C, D, E) Cx46-/− adult mice. (A, B) Spermatogenesis appeared normal in WT and Cx50-/− mice testis sections in which pachytene spermatocytes (P) and dividing secondary spermatocytes are identified (II*). (C, D). However, Cx46-/− testis sections showed numerous tubules with plentiful apoptotic cells particularly
pachytene (P) and diplotene spermatocytes involved in the meiotic division (arrows). In other stages of the cycle, apoptotic spermatids (std) were observed. (E) The lumen was collapsed in some tubules while in others cellular debris or clusters of apoptotic cells (wide arrow) were observed. (F) A histogram of the quantification of immunoperoxidase Apostain-labelled cells is presented. The bars represent the mean \( \pm \) SEM of Apostain-positive cells counted in 25 tubules from three different mice per experimental group. Apostain-labelled cells were significantly more numerous in Cx46-/- than in WT seminiferous tubules (**P<0.005 Cx46-/- vs WT). The number of labelled cells in WT and Cx50-/- mice did not significantly differ. (G) A histogram of nucleosome release measured by cell death detection ELISA in the cytoplasmic fraction of WT, Cx46-/- and Cx50-/- mice tubule-enriched fractions obtained is shown. The data are expressed in optical density at 410 nm and the bars represent the mean \( \pm \) SEM of measurements in three different mice per experimental group. The increase in nucleosome release in Cx46-/- is significantly different (*P<0.05) compared to WT but not between WT and Cx50-/-.. (H-L) Immunoperoxidase labelling of cells in apoptosis with Ab F7-26 (Apostain) is shown. Apostain-positive spermatogonia (g), zygotene (Z), pachytene (P) and diplotene (Di) spermatocytes and spermatids (std) were found in comparable quantities in (H) WT and (I) Cx50-/- mice. (J, K, L) In Cx46-/- mice, Apostain labelling involved spermatogonia (g) and spermatids (std). In addition, zygotene (Z), pachytene (P) and diplotene spermatocytes (Di) were labelled in larger quantities in Cx46-/- than in WT and Cx50-/- mice. (M-R) Bouin’s-fixed Periodic Acid Shiff (PAS)-stained epididymis paraffin sections from (M, N) WT, (O) Cx50-/- and (P, Q, R) Cx46-/-mice are shown. The (M) body and (N, O) tail of the epididymides of (M, N) WT and (O) Cx50-/- exhibited similar histological and both contained spermatozoa (spz) in comparable amounts. However, (P, Q) the epididymides from
Cx46-/- showed spermatozoa in reduced quantities. (P) A cluster of apoptotic cells reminiscent of the one shown in the testis in Figure 3E is identified (wide arrow). Apoptotic spermatocytes and other young germ cells are identified by arrows in the (P) head, (Q) body and (R) tail of Cx46-/- mice epididymides. A-E: X 720.; HI and KO: X 860; PR and J: X 950.

Figure 7. (A-C) The seminiferous tubule-enriched samples were subjected to RT-PCR. The (A) Cx46, (B) Cx50 and (C) Cx43 mRNA levels measured in normal adult mink were compared to those in mink with AIO in February (Feb.) and March (Mar.). The data in arbitrary units are expressed as the mean \( \bar{x} \) (SEM) of three independent experiments and normalised to the normal value for each month. The differences measured between normal mink and mink with AIO were statistically significant for (A) Cx46mRNA (##P<0.00001 Feb. AIO vs Feb. Normal; *P<0.05 Mar. AIO vs Mar. Normal) and for (C) Cx43mRNA (*P<0.05 Feb. AIO vs Feb. Normal, ##P<0.00001 Mar. AIO vs Mar. Normal). (B) The differences in Cx50 mRNA levels measured in normal mink and mink with AIO show no significant difference. Representative Western blots of (D) Cx46, Cx50 and Cx43 in normal adult mink and mink with AIO in February and in March are shown. The quantification of (E) Cx46, (F) Cx50 and (G) Cx43 protein levels measured in normal mink and mink with AIO are provided. The values are the mean \( \bar{x} \) SEM of four independent experiments expressed in arbitrary units were normalised to the 51kDa Cx46 for February; the 51kDa Cx50 for February and total Cx43 for February. The myosin light chain (MLC) was used as the internal loading control and did not significantly change in the different experimental conditions of the study. Significant differences were measured in the junction protein levels between normal mink and mink with AIO: Cx46, February: 51kDa band: @P<0.02 AIO vs
Normal, 68kDa band: *P<0.05 AIO vs Normal; March: 51kDa band: *P<0.05 AIO vs Normal, 68kDa band *P<0.05 AIO vs Normal; Cx50, February: 51kDa band *P<0.05 AIO vs Normal, March: 60kDa band †P<0.03 AIO vs Normal; Cx43: February: +P<0.01 AIO vs Normal, March: ++P<0.001 AIO vs Normal. Immunoperoxidase labelling with anti-Cx46 in (H) February and (I, J) March and with anti-Cx50 in (K) February and (L-N) March of orchitic adult mink testis paraffin sections respectively. (H) Cx46 labelling (open arrowhead) is seen amongst Sertoli cells and Sertoli cell-Sertoli cell (S) and Sertoli cell-spermatogonia (g) and spermatocytes (P) contacts. (I, J) The membrane of vacuoles (vac) remaining behind the exfoliation of germ cells is Cx46-positive. (I) Some of these vacuoles contain cellular debris (deb). (K) Giant cells remaining within or being released from the seminiferous epithelium are identified (Gi). The cells within the giant cell (Gi*) shows signs of apoptosis. (L) Cx50 labelling (open arrowhead) is observed amongst Sertoli cells (S), Sertoli cell contacts to germ cells as well in the perinuclear zone of pachytene spermatocytes (P) in the early phase of AIO but not in (N) tubules where destruction is massive in the late phase of the disease. (M) Cx50 labelling is seen in the wall of a blood vessel. X 860.
Table 1. Specific primers designed for mouse and mink Cx46, Cx50 and Cx43.

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Table 2. Summary of the changes in junction proteins in Cx46/- and Cx50/-.

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