REPORT

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Testin and actin are key molecular targets of adjudin, an anti-spermatogenic agent, in the testis

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Earlier studies have shown adjudin to cause aspermatogenesis by depleting virtually all germ cells from the seminiferous epithelium, leading to transient infertility; spermatogenesis and fertility were re-established several weeks later after germ cell proliferation and differentiation were reinitiated by spermatogonia. While adjudin is known to exert its initial effects at the apical ectoplasmic specialization (a testis-specific atypical anchoring junction), thereby perturbing spermatid adhesion, its molecular target(s) at this site is not known. Herein, we report the production of a specific antibody against adjudin after this compound was conjugated to an adjuvant (i.e., keyhole limpet hemocyanin) to maximize immune response in rabbits. This antibody was utilized for co-immunoprecipitation by using an affinity resin to pull-down the binding partners of adjudin. Using this approach coupled with mass spectrometry and immunoblotting, we show testin (a protein largely restricted to the apical ES in the adult testis) and actin-myosin to be molecular targets of adjudin. These findings provide a platform for future functional studies, not only to better understand the molecular mechanism behind adjudin-induced germ cell loss from the seminiferous epithelium, but also to understand the molecular basis of spermiation.

Introduction

Adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is an anti-spermatogenic compound being actively investigated as a male contraceptive for potential human use.[1,2] In contrast to hormonal approaches which disrupt spermatogenesis by interfering with the hypothalamic-pituitary-testicular axis,[3-5] adjudin exerts its effects behind the blood-testis barrier (BTB) in a specialized microenvironment known as the adluminal compartment. The most obvious phenotype following administration of adjudin to adult rats is the loss of developing germ cells from the seminiferous epithelium, most notably elongating/elongated spermatids, followed by round spermatids and spermatocytes but not spermatogonia residing outside of the BTB.[1,2,6] This leads to a transient loss in fertility; fertility is restored after spermatogonia reinitiate germ cell proliferation and differentiation and cells repopulate the seminiferous epithelium.[6] While adjudin is known to exert its initial effects at the apical ectoplasmic specialization (apical ES, a testis-specific anchoring junction found between Sertoli cells and step 8–19 spermatids in the rat testis), the molecular target(s) of adjudin remains unknown. This important information, if it were available, would provide additional insight on the molecular basis behind the restricted action of adjudin in the testis, which is still not completely understood. While both acute and subchronic toxicity studies performed by licensed toxicologists following FDA guidelines have shown adjudin to exert its effects primarily in the testis by depleting germ cells from the seminiferous epithelium without affecting cell adhesion in other organs,[7] adjudin was not specifically uptaken by the testis. Instead, [^{3}H]-adjudin was distributed rather uniformly amongst different organs, less than 0.05% of [^{3}H]-adjudin was recovered from the testis, and [^{3}H]-adjudin was metabolically cleared by ~24 hours after administration.[6] If our hypothesis—which asserts that adjudin affects adhesion in the testis because of the presence of this unique structure—holds ground, then adjudin should interact with an apical ES-specific protein(s) to trigger ES disassembly. This is the question that we address in this study.

The ES is an actin-based cell-cell anchoring junction that is found at two important sites within the seminiferous epithelium: (1) at the Sertoli cell-elongating/elongated spermatid juncture (defined as the apical ES) and (2) at the Sertoli-Sertoli cell interface at the BTB (defined as the basal ES).[8,9] Once the apical ES is assembled at the Sertoli cell—step 8 spermatid interface during spermiogenesis, it becomes the only anchoring device to persist until late spermatids are released from the epithelium at spermiation.[8,10] The basal ES at the BTB, on the other hand, co-exists with other junction types, namely tight junctions (TJs), desmosomes and gap junctions (GJs).[11] It is also worth noting that both the apical and the basal ES share nearly identical ultrastructural

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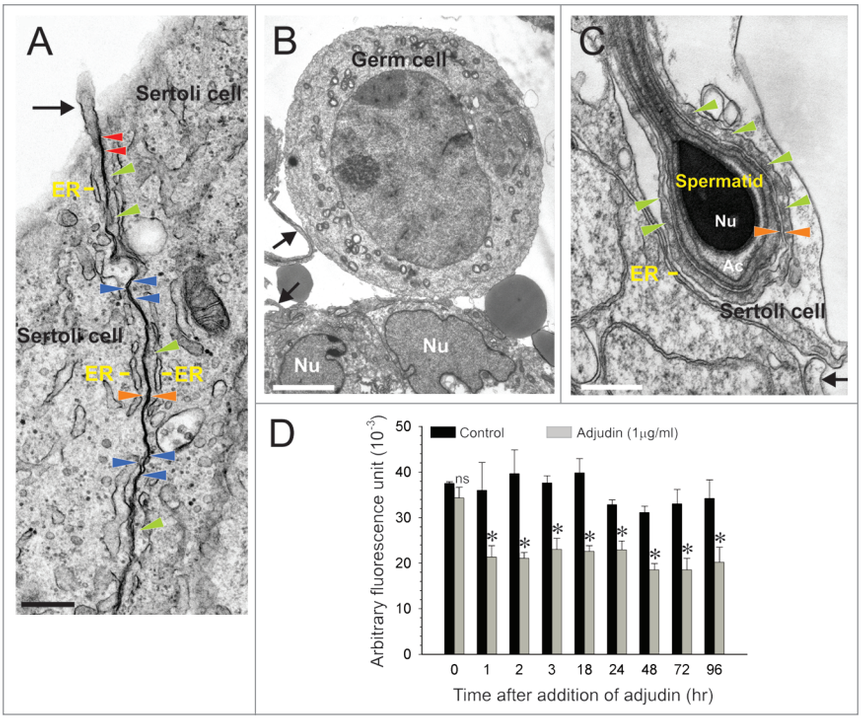

Figure 1. Use of Sertoli and germ cell co-cultures to assess the effects of adjudin on disrupting germ cell adhesion in vitro. (A) This is a typical Sertoli cell epithelium in which cells were cultured for 4 days. A functional Sertoli cell barrier, manifested by the presence of TJs (see blue arrowheads) and basal ES, is visible between adjacent Sertoli cells. The basal ES is typified by the presence of actin filament bundles (see green arrowheads) sandwiched in between cisternae of endoplasmic reticulum (ER) and apposing Sertoli cell plasma membranes (see orange arrowheads). Desmosomes, typified by the presence of electron-dense material, are also visible (see red arrowheads). (B) A germ cell attaches to the Sertoli cell epithelium ~48 hours after seeding germ cells (Nu, Sertoli cell nucleus). (C) This is a step 9–10 spermatid with a functional apical ES, typified by the presence of actin filament bundles (see green arrowheads) sandwiched in between cisternae of ER and apposing Sertoli cell-spermatid plasma membranes (see orange arrowheads). Both the acrosome (Ac) and condensed general material within the nucleus (Nu) are visible. Microvilli (A–C) are also seen in all micrographs (see black arrows). (D) Germ cells, such as those shown in (B and C) were fluorescently labeled and added onto the Sertoli cell epithelium (A) at time 0. After Sertoli-germ cell adhesion was established, adjudin (1 μg/ml) was added into triplicate wells per time point to assess its effects on germ cell adhesion (see Materials and Methods). A loss in germ cell adhesion was detected 1 hour (hr) after treatment when the amount of fluorescence remaining in co-cultures was quantified by cytofluorometry. Error bars represent mean ± SD from at least three different experiments. *p < 0.05; ns, not significant. (two-way ANOVA followed by Tukey's post-hoc test). Bar in A = 0.5 μm, B = 4 μm, C = 0.3 μm.

features when examined by electron microscopy. These are typified by tightly packed bundles of non-contractile actin filaments that are sandwiched in between cisternae of endoplasmic reticulum and the Sertoli cell plasma membrane.[8,12-14] Interestingly, these ultrastructural features are not found within spermatids so that the apical ES can be 'loosely' defined as a hemi-junction; we say this because even though the ES is a hemi-junction, its adhesion in vitro is stronger than that of the desmosome which is present between Sertoli cells and all germ cells up to, but not including, step 8 spermatids.[15,16] Moreover, not all multi-protein complexes found at the apical ES (e.g., α6β1-integrin-laminin-333 and junctional adhesion molecule-C-zonula occludens-1) are necessarily found at the basal ES and vice versa.[8,17,18] Lastly, many proteins such as cadherin, coxsackievirus and adenovirus receptor (CAR), and connexin 43 that constitute functionality to other junction types (i.e., adherens junctions, TJs and GJs, respectively) are also found at the apical ES.[11,16,19-21]

Testin is yet another example of a protein found predominantly at the apical ES.[22-27] Interestingly, testin expression was shown to be transiently induced several-fold whenever apical ES function was disrupted, leading to aspermatogenesis and whenever spermatogenesis was programmed to return to normal.[28] Studies by gene profiling using testes from rats treated with adjudin versus methylcellulose (control) have also identified testin as one of the most induced genes;[29] the only other gene to be induced more than testin was wingless-type MMTV integration site family, member 4 (Wnt4) (Mruk DD, Cheng CY, unpublished observation). Similarly, gamendazole, an analog of adjudin, was also shown to stimulate testin expression in the adult testis by 3.2-, 17.6- and 52.4-fold versus control rats by 4, 12, and 24 hours, respectively, in a gene profiling study.[53] Collectively, these findings illustrate that the apical ES is a testis-specific hybrid junction and that it is the primary cellular target of adjudin. Herein, we report findings emanating from a series of experiments that identify the molecular targets of adjudin at the apical ES. These results should be helpful in the future study of adjudin (or its analogs) as a non-hormonal male contraceptive. These findings may also help us better understand the process of spermiation.

Results

Effects of adjudin on germ cell adhesion to Sertoli cells in vitro. As previously reported, adjudin is known to affect the adhesion of germ cells to Sertoli cells in the seminiferous epithelium.[19,28,30] We have established an in vitro assay using Sertoli-germ cell co-cultures to examine the effects of adjudin on germ cell adhesion. This assay also aims to facilitate rapid screening of second and third generation adjudin analogs (or other compounds having a similar mechanism of action) in order to avoid excessive use of laboratory animals, which would be needed to establish initial efficacy. In brief, Sertoli cells were cultured at 0.5 x 10^6 cells/cm^2 for 4 days to allow the establishment of a functional TJ permeability barrier as assessed by transepithelial electrical resistance (TER) measurements.[31] As shown in **Figure 1A**, TJs (see blue arrowheads) were found to co-exist with basal ES, a structure typified by the presence of actin filament bundles (see green arrowheads) sandwiched in between

cisternae of endoplasmic reticulum (ER) and apposing Sertoli cell plasma membranes (see orange arrowheads). Desmosomes (see red arrowheads), characterized by the presence of electron-dense material, were also found at the Sertoli-Sertoli cell interface (**Fig. 1A**). Together, these junctions constitute the Sertoli cell barrier/BTB (**Fig. 1A**). On day 4, germ cells were isolated from adult rat testes, seeded onto the Sertoli cell epithelium and allowed to establish adhesion with Sertoli cells (**Figs. 1B and C**). **Figure 1C** shows adhesion between a step 9–10 spermatid and a Sertoli cell. A functional ES, typified by the presence of actin filament bundles (see green arrowheads) sandwiched in between the ER and apposing Sertoli cell-spermatid plasma membranes (see orange arrowheads) is visible in this micrograph as well. Also, Sertoli cells cultured in vitro were found to have microvilli (**Fig. 1A–C** and see black arrow). The acrosome (Ac) and spermatid nucleus (Nu) are noted in **Figure 1C**. An in vitro assay was performed by fluorescently labeling germ cells and allowing them to adhere to the Sertoli cell epithelium, after which adjudin (1 μg/ml) was added. Adjudin was shown to perturb germ cell adhesion within 1 hour (**Fig. 1D**). Since adjudin is less effective in disrupting adhesion between Sertoli cells and step 1–7 spermatids, spermatocytes and spermatogonia,[32,33] it could not detach all germ cells from the Sertoli cell epithelium within 96 hours (**Fig. 1D**). Alternatively, a higher concentration of adjudin may be needed to dislodge these germ cells. These findings are consistent with earlier in vivo studies, which demonstrated round spermatids and spermatocytes to detach from the epithelium by ~3 and 6.5 days, respectively, versus elongating/elongated spermatids by ~6 hr, after adjudin treatment.[32]

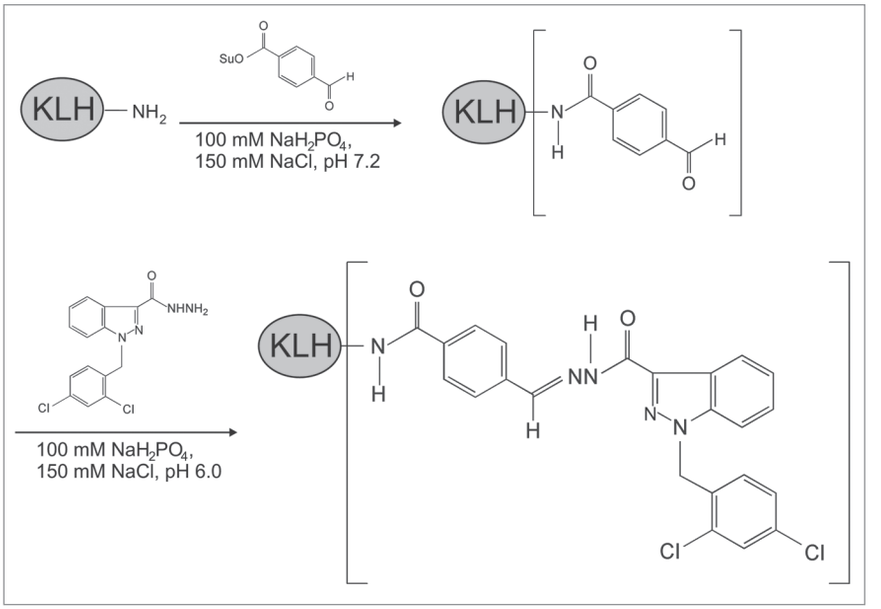

Figure 2. Conjugation of adjudin to KLH (keyhole limpet hemocyanin, an adjuvant), and its use as an immunogen for anti-adjudin antibody production. KLH was first reacted with SFB (succinimidyl 4-formylbenzoate, a heterobifunctional crosslinker) to generate a benzaldehyde at its N-terminus. This modified KLH was then reacted spontaneously in physiological buffer with the hydrazide group in adjudin, forming a stable KLH-adjudin conjugate via a hydrazone linkage.

Specificity of the anti-adjudin antibody. To identify the molecular target(s) of adjudin in the seminiferous epithelium, affinity chromatography was used. For this reason, we raised a specific antibody against anti-adjudin. **Figure 2** illustrates the conjugation scheme for linking adjudin to keyhole limpet hemocyanin (KLH) where KLH served as an adjuvant. KLH was initially modified with succinimidyl 4-formylbenzoate (SFB) to incorporate a benzaldehyde moiety at its N-terminus. This, in turn, reacted readily with the hydrazide group of adjudin (**Fig. 3A**) to form a stable KLH-adjudin conjugate via the hydrazone linkage (**Fig. 2**). This conjugate was then used as an immunogen for anti-adjudin antibody production in two rabbits, one male and one female.[32] As shown in the adjudin radioimmunoassay (RIA), adding increasing concentrations of unlabeled (i.e., cold) adjudin generated a complete displacement curve with a 50% displacement at ~10 ng and a sensitivity of ~0.35 ng adjudin (**Fig. 3C**). Because lonidamine is structurally similar to adjudin (i.e., presence of carboxylic acid in the indazole ring of lonidamine instead of carbohydrazide in adjudin, **Fig. 3B versus 3A**), lonidamine generated a parallel displacement curve. However, lonidamine failed to completely displace the binding of [^{3}H]-adjudin to its antibody, illustrating the specificity of this anti-adjudin antibody. Equally important, adjudin is known to share structural similarities with other steroids such as testosterone and estradiol-17β. While [^{3}H]-adjudin was found to bind to dextran-coated charcoal, both testosterone and estradiol-17β at up to 100 ng per assay tube failed to compete with the binding of [^{3}H]-adjudin to its antibody, illustrating the lack of cross-reactivity. In conclusion, this anti-adjudin antibody is highly specific to adjudin.

Anti-adjudin antibody protects the seminiferous epithelium from the destructive effects of adjudin on germ cell depletion in vivo. Competitive binding assays based on the use of *Staphylococcus aureus* cells (formalin fixed) instead of dextran-coated charcoal absorption also confirmed that [^{3}H]-adjudin bound to adjudin antiserum and that this binding was displaced in the presence of excess unlabelled adjudin (**Fig. 4A**). The use of pre-immune serum in place of adjudin antiserum yielded no detectable [^{3}H]-adjudin binding (**Fig. 4A**). These results were confirmed and expanded in the next in vivo experiment, which illustrated that adjudin-mediated germ cell loss from the seminiferous epithelium was effectively blocked by intratesticular injection of anti-adjudin IgG (**Fig. 4Bi–iii versus**). However, injection of normal rabbit IgG (isolated from pre-immune serum) could not block the adverse effects of adjudin on Sertoli-germ cell adhesion (**Fig. 4Biii**). **Figure 4Bi** is a cross-section of the testis from control rats and **Figure 4Bii** is a cross section of the testis from rats 21 days after being treated with adjudin (50 mg/kg b.w., by

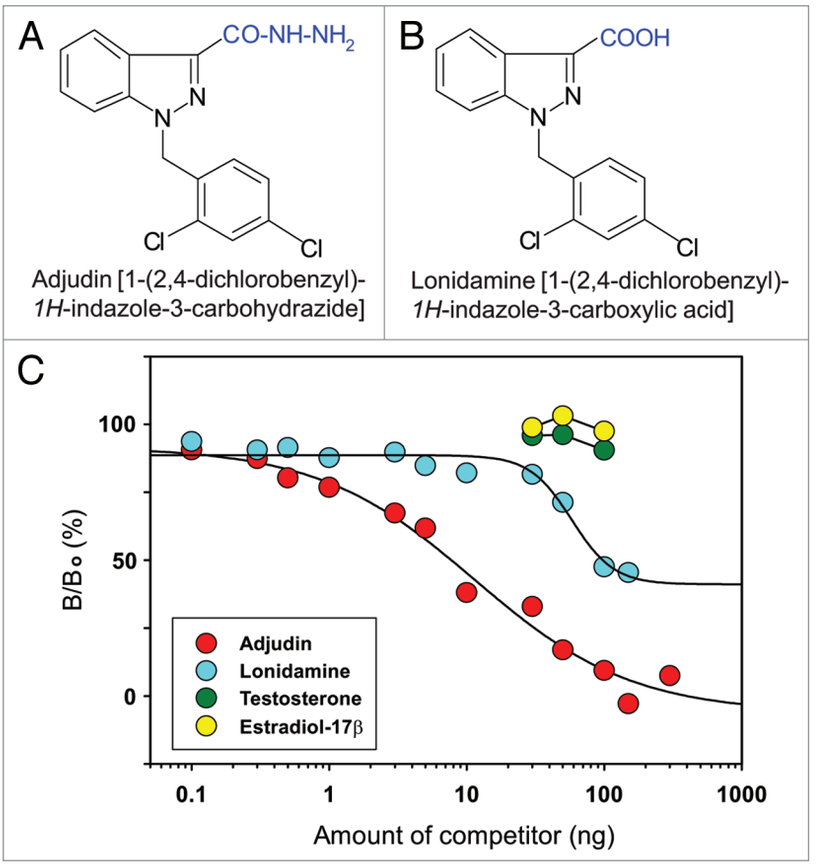

Figure 3. Specificity analysis of anti-adjudin antiserum. Structural formulae of adjudin (A) and lonidamine (B). (C) Displacement of [^{3}H]-adjudin binding to anti-adjudin antiserum by unlabelled (i.e., cold) adjudin, lonidamine, testosterone or estradiol-17β. Adjudin competed with the binding of [^{3}H]-adjudin to its antibody. While lonidamine shares structural similarities with adjudin, it generated a parallel but incomplete displacement curve. Both testosterone and estradiol-17β failed to compete with the binding of [^{3}H]-adjudin to its antibody.

gavage). Because entry of anti-adjudin IgG (Mr 150 kDa) into the seminiferous epithelium was likely excluded by the BTB,[34,35] we believe that anti-adjudin encased the periphery of seminiferous tubules, thereby preventing the entry of adjudin into the adluminal compartment. This protected germ cell adhesion from the destructive effects of adjudin.

Actin-myosin and testin are molecular targets of adjudin. Earlier studies from our laboratory have shown adjudin to exert its effects mostly at the apical ES,[2,19,36] an actin-based hybrid junction found uniquely in the testis.[8,37] Moreover, studies using immunofluorescence[9,38] and electron microscopy[39] have shown the actin cytoskeleton to be affected by adjudin. To confirm that actin is a cellular target of adjudin, we opted to use a biochemical approach (i.e., co-immunoprecipitation). In brief, anti-adjudin IgG was allowed to form a stable immunocomplex with Protein A-Sepharose, which was packed onto an Econo-Column. Thereafter, lysates of testes obtained from rats treated with adjudin were re-circulated through the column for several hours. This allowed adjudin interacting proteins to form a stable complex with the anti-adjudin IgG-Protein A Sepharose. After washing the column extensively, proteins were eluted with a low pH buffer. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their identities were determined by mass spectrometry (**Fig. 5**) and immunoblotting using corresponding specific antibodies (**Figs. 6 and 7**). Using these approaches, actin (42 kDa) at the apical ES was shown to be one of the primary targets of adjudin (**Figs. 5 and 6**). While adjudin may also target actin at the basal ES, we do not think this is the case because BTB integrity was unaffected during this time,[11] and it remained unaffected until ~6 weeks following adjudin treatment when the BTB was found to be transiently disrupted.[40] Myosin (Mr ~160 kDa) was also identified as an adjudin target in the testis (**Fig. 5**).

In addition to actin and myosin, testin (35 and 37 kDa) was also pulled-down as a molecular target of adjudin as demonstrated by co-immunoprecipitation and immunoblotting (**Fig. 7A**). The interaction of adjudin with actin and testin was found to be specific since unlabeled adjudin displaced the binding of [^{3}H]-adjudin to actin or testin IgG in competitive binding assays (**Figs. 6B and 7B**). This latter finding is not entirely unexpected because testin is a constituent of the apical ES,[27,41] and its presence at the apical ES was confirmed by a study using autoradiography at the electron microscopy level.[26]

Discussion

As discussed above, the apical ES is a unique junction that is restricted to the Sertoli cell-elongating/elongated spermatid interface. It is typified by the presence of actin filament bundles sandwiched in between cisternae of the ER and apposing Sertoli cell-spermatid plasma membranes.[9,13,19,42] This unique arrangement of actin filament bundles appears to contribute to the remarkable adhesive strength of the apical ES since the force required to pull a pre-step 8 spermatid was found to be weaker than that required to pull a post-step 8 spermatid from the Sertoli cell epithelium in vitro as assessed by a micropipette pressure transducing system.[15] Specifically, the mean force that was required to detach spermatocytes, pre-step 8 spermatids and post-step 8 spermatids from the Sertoli cell epithelium was 5.25 x 10^{-7} pN, 4.73 x 10^{-7} pN and 8.82 x 10^{-7} pN,[15] respectively, illustrating that the apical ES is almost twice as stable as the desmosome in terms of adhesion. Moreover, the inclusion of adjudin into these co-cultures significantly weakened the apical ES; hence, less force was needed to pull post-step 8 spermatids from the Sertoli cell epithelium.[33] These in vitro findings are important because they confirm earlier in vivo observations which illustrated the apical ES to be the primary cellular target of adjudin in the seminiferous epithelium.[32] Unfortunately, the molecular mechanism behind these observations continues to remain largely

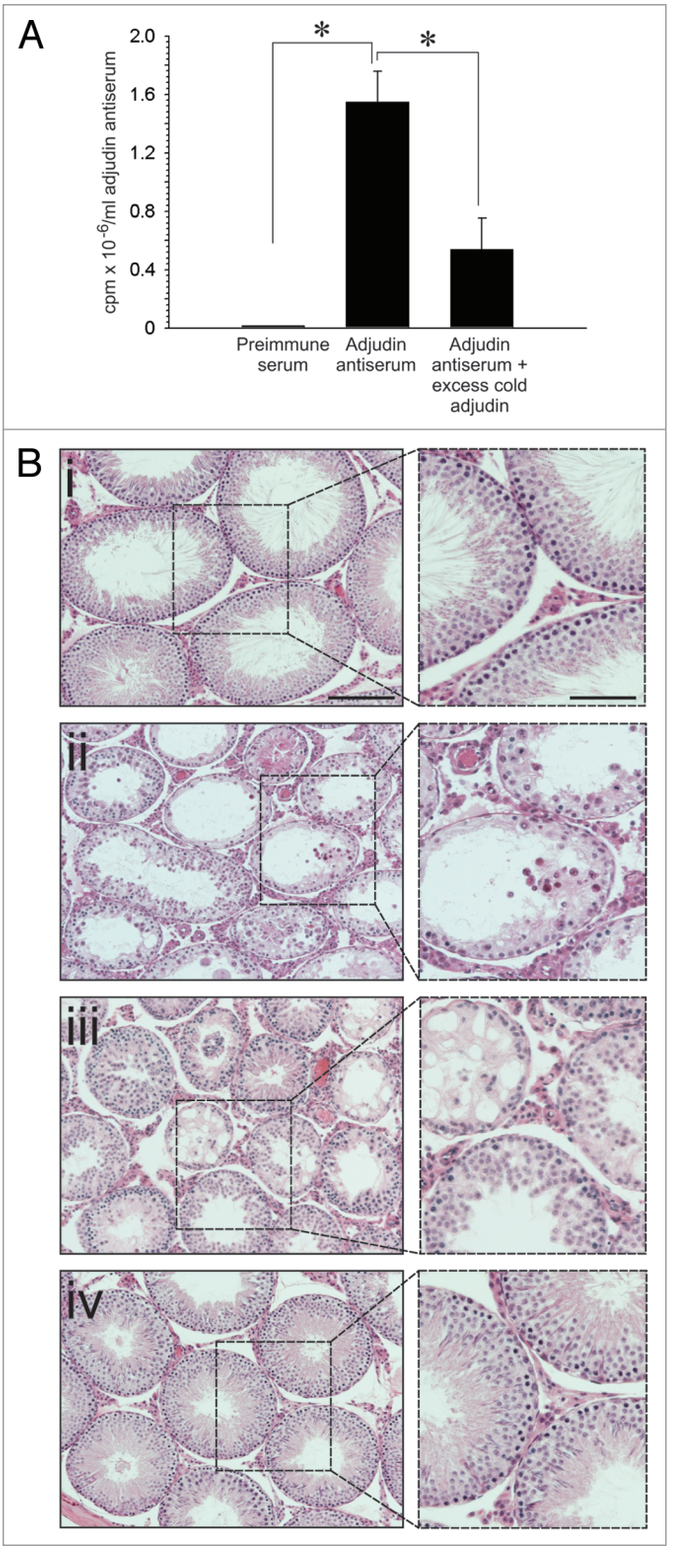

Figure 4. A study to assess the ability of the anti-adjudin antibody to protect adjudin-induced germ cell loss from the seminiferous epithelium in rat testes. (A) Adjudin competed with the binding of [^{3}H]-adjudin to its antibody in a competitive binding assay. (B) Anti-adjudin IgG blocked the effects of adjudin on Sertoli-germ cell adhesion. (i) Cross-section of the control testis. (ii) Cross-section of the testis 21 days after treatment with adjudin (50 mg/kg b.w.). (iii) Cross-section of the testis 21 days after treatment with non-immune rabbit IgG and adjudin (50 mg/kg b.w.). (iv) Cross-section of the testis 21 days after treatment with anti-adjudin IgG and adjudin. All cross-sections were stained with H&E. Boxed areas in (i–iv) represent magnified views, and these are shown to the right of each image. Bar in (Bi) [also applies to (Bii–iv)] = 125 μm; bar in magnified views = 75 μm. Error bars represent mean ± SD from four different experiments. *$p < 0.05$. (two-way ANOVA followed by Tukey's post-hoc test).

unknown. Herein, we utilized a specific anti-adjudin antibody and co-immunoprecipitation to show that actin-myosin and testin bind adjudin, which destabilizes actin filament bundles as visualized by electron microscopy[39] and leads to premature germ cell loss (i.e., pseudo-spermiation). This postulate was further supported by recent findings that showed a reduction in epidermal growth factor receptor pathway substrate 8 (EPS8, a protein that caps the barbed ends of and bundles actin filaments[37,43,44]) and a mis-localization of actin related protein 3 (ARP3, a component of the ARP2/3 complex, which together with neuronal Wiskott-Aldrich syndrome protein [N-WASP] and ARP2/3 complex subunits 1 to 5 [ARPC 1–5] form a regulatory complex to induce branching of the actin network[37,45,46]) at the apical ES. The net result of these changes is the destabilization of cell adhesion and loss of germ cells.

Similar to lonidamine, adjudin is a small non-steroidal molecule,[47] and studies have shown the hypothalamic-pituitary-testicular axis not to be affected by adjudin (or its analog, gamendazole) because serum luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone levels did not change.[28,48] The only exception to this finding is that elevated LH and FSH levels were noted in men 1 and 3 weeks after lonidamine treatment, but testosterone, prolactin and thyroid-stimulating hormone (TSH) levels all remained unchanged.[49] In another study, five cancer patients being treated with lonidamine for 4–8 weeks exhibited a significant decrease in their serum testosterone levels.[50] Thus, although lonidamine and adjudin are structurally similar, their mechanisms of action are somewhat different. Also, it remains to be determined if adjudin has any chemotherapeutic properties, similar to lonidamine. The fact that adjudin can be pulled-down with dextran-coated charcoal seemingly suggests that it shares physico-chemical properties, at least partly, with steroids. Furthermore, the anti-adjudin antibody was shown to be highly specific to adjudin; it did not recognize testosterone or estradiol-17β, and despite its structural similarity to lonidamine, it only partially cross-reacted with lonidamine. Based on the findings reported in this study, we believe that adjudin binds to testin at the apical ES, which in turn affects cell adhesion mediated by several multi-protein complexes (e.g., integrin-laminin, cadherin-catenin, JAM-ZO-1, CAR-ZO-1 or nectin-afadin). EPS8 and ARP3 may also be involved, that is, they may regulate some aspect of protein internalization, which would be needed for apical ES disassembly.

In this context, the precise role of testin in these cellular events is not yet known. What is known is that testin is a signaling molecule whose expression surges during apical ES disassembly[23,28,51] (its level is also highest at late stage VIII of the seminiferous epithelial cycle[27]) and that testin binds actin strongly

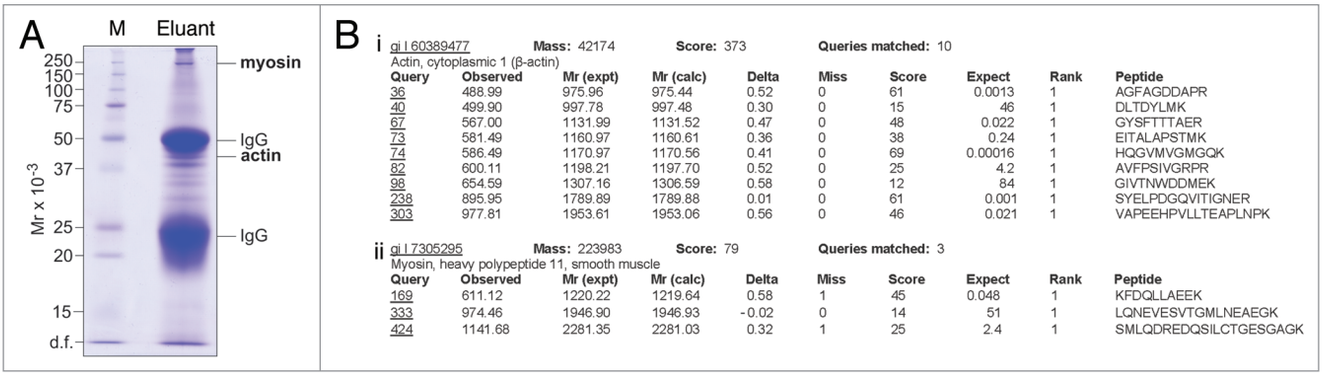

Figure 5. Identification of actin and myosin as molecular targets of adjudin in the rat testis by co-immunoprecipitation and mass spectrometry. (A) Actin and myosin were found to bind adjudin as determined by co-immunoprecipitation using affinity chromatography with an anti-adjudin IgG-Protein A resin, coupled with SDS-PAGE, Coomassie blue gel staining and mass spectrometry. M, molecular weight marker. (B) Sequences corresponding to actin (i) and myosin (ii) as obtained by mass spectrometry.

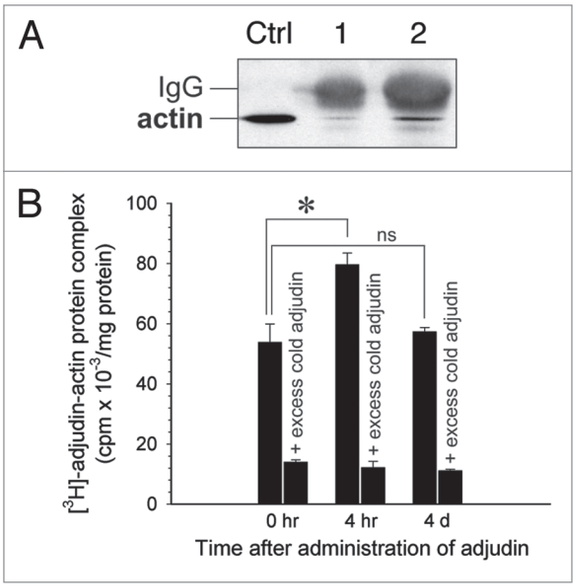

Figure 6. Characterization of actin as a molecular target of adjudin. (A) Adjudin binds to actin (42 kDa) as determined by co-immunoprecipitation. Aliquots (lane 1, 10 μl; lane 2, 30 μl) of the eluant (**Fig. 5A**) were resolved by SDS-PAGE and proteins were transferred to nitrocellulose for immunoblotting using anti-actin IgG. Ctrl, testis lysate (20 μg). (B) The interaction of adjudin with actin was specific since unlabeled (i.e., cold) adjudin displaced the binding of [^{3}H]-adjudin to actin IgG in a competitive binding assay. Error bars represent mean ± SD from three different experiments. *$p < 0.05$; ns, not significant. (Student's t-test).

(Mruk DD and Cheng CY, unpublished observations). Testin expression is also largely restricted to the testis, as well as to the ovary.[23,24] This brings into question the exact role of testin in actin dynamics. While actin is present in all cells, adjudin does not affect adhesion in other organs.[6,7] Although a speculation, testin may be responsible for the non-contractile nature of actin at the apical ES,[11] and non-contractility (i.e., rigidity) of actin filaments at the apical ES may also make this structure more susceptible to compounds such as adjudin by a yet unknown mechanism. Alternatively, the testin-actin protein complex may be functioning as a platform to recruit EPS8 and ARP3 (or other proteins) to the apical ES. The observation that adjudin also binds myosin, a motor protein that itself binds to actin, is also important because myosins are responsible for actin-based motility and endocytosis.[52]

In summary, we have identified actin-myosin and testin as molecular targets of adjudin at the apical ES. Because testin is an ES protein, these findings explain the restricted action of adjudin in the testis. Adjudin apparently exerts its effects by binding to testin since adjudin[28,29] or its analog, gamendazole,[53] are known to upregulate testin expression in the testis, in turn perturbing actin, affecting adhesion and resulting in germ cell loss. Nevertheless, additional research is needed to better understand adjudin's mechanism of action in the testis.

Materials and Methods

Animals. Sprague-Dawley (outbred) rats were purchased from Charles River Laboratories. New Zealand white rabbits were obtained from Harlan Laboratories. All animals were housed at The Rockefeller University Comparative Bioscience Center at 22 ± 1°C with constant light:dark cycles of 12 hour:12 hour with free access to standard chow and water. The use of rats and rabbits was approved by The Rockefeller University Animal Care and Use Committee with protocol numbers 03040, 06018 and 09016.

Electron microscopy of Sertoli-germ cell co-cultures. Sertoli cells were isolated from the testes of 20-day-old rats.[54,55] By this postnatal day, Sertoli cells had ceased to divide and were fully differentiated.[56] Also, these cells are known to functionally mimic Sertoli cells isolated from adult rat testes.[57,58] Sertoli cells were plated on Matrigel™-(BD Biosciences) coated 60-mm dishes at 0.5 x 10^6 cells/cm^2 and cultured in serum-free Dulbecco's modified Eagle medium: Ham's nutrient mixture F-12 (DMEM/F-12 at 1:1, [v/v]) supplemented with growth factors and antibiotics as described.[54,55] Approximately 36 hours after plating, cultures were treated with a hypertonic buffer (20 mM Tris pH 7.4 at 22°C) to lyse contaminating germ cells,[59]

resulting in a Sertoli cell purity of >98%. Also, there was negligible contamination by Leydig and peritubular myoid cells when cell specific markers were used for RT-PCR and immunoblotting experiments as previously described.[60] A functional TJ permeability barrier and ultrastructural features corresponding to TJs, basal ES and desmosomes which mimicked the BTB in vivo when examined by electron microscopy were also noted in these Sertoli cell cultures by day 4. On day 4, total germ cells were isolated from adult rat testes.[61] Successive passages through glass wool were used to remove step 12–19 spermatids, and isolated germ cells were then incubated with 1 μM gelsolin (an actin-severing protein,[62] Sigma-Aldrich) in DMEM/F-12 for 15 min to remove residual actin filaments which may have remained attached to spermatids during the isolation procedure. Germ cell purity was >95% with negligible somatic cell contamination,[61] and germ cells were co-cultured with Sertoli cells within 1 hour of their isolation. On day 6 (i.e., ~48 hours after plating germ cells onto the Sertoli cell epithelium), co-cultures were fixed in 2.5% glutaraldehyde [v/v]/2.5% paraformaldehyde [w/v] in 0.1 M sodium cacodylate buffer pH 7.5 at 22°C. Sertoli cells cultured alone for 6 days were also processed for electron microscopy as described.[26] Cells were post-fixed in 1% OsO_4 [v/v] in 0.1 M sodium cacodylate buffer and stained with uranyl acetate. After dehydration in ascending grades of ethanol, cells were detached from dishes with propylene oxide and subsequently embedded in EPON (Electron Microscopy Sciences). Silver sections were generated with an ultramicrotome, and images were obtained with a JEOL 100CDXII electron microscope at 80 kV. Electron microscopy was performed at the Bio-imaging Resource Center, The Rockefeller University.

In vitro germ cell binding assays. Sertoli cells isolated from the testes of 20-day-old rats were plated on Matrigel™-coated 24-well dishes (Nunc) at 0.5 x 10^6 cells/cm^2 and cultured in DMEM/F-12 for 4 days as previously described with a hypotonic treatment performed ~36 hours after plating.[59] Thereafter, germ cells isolated from the testes of 90-day-old rats were fluorescently labeled with 1,1'-dioctadecyl-6,6'di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine [SP-DiIC_{18}(3), Molecular Probes/Invitrogen)]. Successive passages through glass wool were employed to remove step 12–19 spermatids. Germ cells (1 x 10^6 cells/1 ml) were labeled with SP-DiIC_{18}(3) [2 μM, diluted from a 20 μM stock solution prepared in DMSO] at 35°C for 5 min. This was followed by an additional incubation at 4°C for 15 min. Germ cell viability was not affected during this labeling procedure, and only viable germ cells were fluorescently labeled. Thereafter, fluorescently labeled germ cells were successively washed with DMEM/F-12, added onto the Sertoli cell epithelium at a Sertoli:germ cell ratio of 1:3, and allowed to adhere to Sertoli cells for 24–36 hours. The following day, co-cultures were washed twice, and DMEM/F-12 containing adjudin (1 μg/ml, diluted from a 1 mg/ml stock solution prepared in ethanol) was added into wells, after which time DMEM/F-12 was no longer changed. At specific time points, co-cultures in triplicate wells were terminated by washing three times to remove unbound germ cells, and fluorescence (530 nm_{EX}/590 nm_{EM}) was quantified with a GENios cytofluorometer (Phenix Research Products).

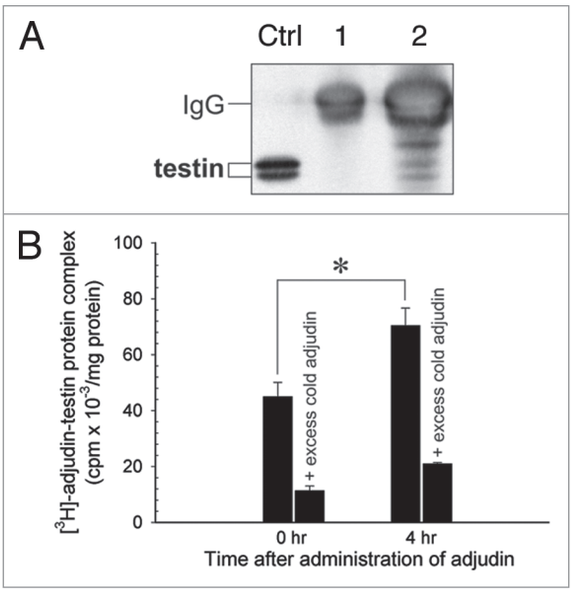

Figure 7. Characterization of testin as a molecular target of adjudin. (A) Adjudin binds to testin (35 and 37 kDa) as determined by co-immunoprecipitation. Aliquots (lane 1, 10 μl; lane 2, 30 μl) of the eluant (**Fig. 5A**) were resolved by SDS-PAGE and proteins were transferred to nitrocellulose for immunoblotting using a monospecific anti-testin antibody.[22,23] Ctrl, testis lysate (100 μg). (B) The interaction of adjudin with testin was specific since unlabeled (i.e., cold) adjudin displaced the binding of [^{3}H]-adjudin to testin IgG in a competitive binding assay. Error bars represent mean ± SD from three different experiments. *p < 0.05. (Student's t-test).

Production of adjudin antiserum. Adjudin antiserum was produced by a standard protocol.[63] In brief, adjudin was cross-linked to keyhole limpet hemocyanin (KLH) as follows. First, KLH was reacted with succinimidyl 4-formylbenzoate (SFB, Pierce Biotechnology) to yield an aldehyde group at its N-terminus. Second, this aldehyde group was reacted readily with the hydrazide group in adjudin to form a stable hydrazone bond. This conjugate with a 1:1 molar ratio of KLH:adjudin served as the immunogen, which was subsequently emulsified in Freund's Adjuvant and injected subcutaneously at three different sites into two New Zealand rabbits (1 male and 1 female, ~4 kg b.w. at the time of immunization) with ~1 μmol adjudin per site (i.e., ~3 μmol adjudin per immunization per rabbit). At 6- and 8-week intervals, animals received a booster injection containing the same amount of immunogen as was used for the initial immunization. Thereafter, blood was collected from the marginal vein in the ear to obtain antiserum.[64] Anti-adjudin IgG was prepared by $(NH_4)_2SO_4$ precipitation and DEAE-chromatography.[64-66] We routinely obtained ~2 mg purified (as assessed by SDS-PAGE and Coomassie blue staining) anti-adjudin IgG per ml antiserum.

Characterization of adjudin antiserum. Adjudin antiserum was partially characterized by two methods. First, adjudin antiserum was used for competitive binding assays. In brief, binding buffer (10 mM NaH_2PO_4, pH 7.4 at 22°C containing 0.15 M NaCl, 0.5% BSA [w/v], 0.2% gelatin [w/v] and 0.05% NaN_3

[w/v]), adjudin antiserum (1:50–1:2,000) and [^{3}H]-adjudin (15 x 10^6 cpm) were combined in reaction tubes to a final volume of 500 μl. Samples were vortexed and incubated at 4°C for 36 hours. Thereafter, *Staphylococcus aureus* (*Sac*) cells (formalin-fixed) were added to separate bound from free [^{3}H]-adjudin, and samples were incubated at 4°C for 1 hour. Samples were centrifuged and radioactivity quantified in pellets. As a control, pre-immune serum was used instead of adjudin antiserum. [^{3}H]-Adjudin (specific activity, 580 mCi/mmol) was obtained from PerkinElmer.[6]

Second, anti-adjudin IgG was administered to adult rats (~300 gm b.w.) to determine if it could protect the testis from the adverse effects of adjudin on Sertoli-germ cell adhesion. Animals were divided into four groups: *group 1*-received anti-adjudin IgG (by intratesticular injection) plus adjudin (50 mg/kg b.w. by gavage; n = 5), *group 2*-received non-immune rabbit IgG plus adjudin (n = 3), *group 3*-received adjudin only (n = 3) and *group 4*-control, received 0.5% methylcellulose [w/v] (n = 3). In brief, 100 μg anti-adjudin or non-immune rabbit IgG suspended in 75 μl sterile PBS, pH 7.4 at 22°C was injected intratesticularly into the right testis with a 29-gauge needle-syringe combination. (Note: the left testis received no injection of IgG). This was followed immediately by oral administration of adjudin (50 mg/kg b.w. suspended at 20 mg/ml in 0.5% methyl cellulose [w/v]) to induce germ cell loss from the seminiferous epithelium.[28,30] This time point was designated as day 0. Thereafter, rats received two additional injections of anti-adjudin or non-immune rabbit IgG (100 μg each) on days 3 and 6. These days were chosen because the most extensive loss of germ cells from the seminiferous epithelium occurred during this time. All animals in this experiment were sacrificed on day 21. Testes were removed, embedded in paraffin and stained with hematoxylin and eosin (H&E).

Adjudin radioimmunoassay (RIA). An adjudin RIA was established as follows. The appropriate antiserum dilution was estimated in pilot experiments using various dilutions ranging from 1:10 to 1:1,000. A 1:50 dilution was selected because it yielded a ~30% binding, which was almost completely displaced by unlabeled adjudin. A standard curve was prepared in triplicate using unlabeled adjudin (or lonidamine) diluted in ethanol ranging from 0.1 to 300 ng per assay tube to assess their ability to displace [^{3}H]-adjudin from binding to the anti-adjudin antibody. RIA buffer (0.05 M Tris-HCl pH 8.0 at 22°C containing 0.1 M NaCl, 0.1% NaN_3 [w/v] and 0.1% gelatin [w/v]), cold adjudin (dissolved in ethanol at 1 mg/ml) and adjudin antiserum (1:50) were combined in reaction tubes to a final volume of 600 μl. Samples were vortexed and incubated at room temperature for 30 min. Thereafter, 100 μl [^{3}H]-adjudin (~2 x 10^4 cpm) was added into each tube, samples vortexed and incubated at 60°C in a shaking water bath. Samples were cooled down at 4°C before adding 300 μl ice-cold dextran-coated charcoal suspension (0.5% activated charcoal [w/v] in RIA buffer containing 0.05% dextran T70 [w/v]) to each tube. Samples were vortexed and placed on an ice-water bath for 10 min, followed by centrifugation at 2,500 *g* for 10 min at 4°C. Supernatants were carefully decanted into scintillation vials and ~5-ml of Ready Safe scintillation fluid (Beckman) was added. Radioactivity was quantified in supernatants with a Packard Tri-Carb 2900TR β-counter. Non-specific binding was assessed by using RIA buffer containing 0.1% gelatin [w/v]. Assays were computer-analyzed by fitting the standard curve to a four-parameter logistic function and unknowns were interpolated from the resultant curve using the software program SigmaPlot (version 12, Systat Software Inc.). The minimal detectable dose was ~0.35 ng adjudin per assay tube and 50% displacement was at 9.5 ng adjudin. The intra- and inter-assay coefficient of variation was 8% and 12%, respectively.

Identification of the cellular targets of adjudin. Adjudin (100 mg/kg b.w.) was administered orally to adult rats (~300 gm b.w., n = 3). Animals were sacrificed 4 hours after administration when the level of adjudin was highest in the testis.[6] Also, this time point was selected because it preceded the loss of elongated spermatids from the seminiferous epithelium which occurred at ~6.5 hours.[32] Testes from three different rats were combined and used to prepare lysate in lysis buffer (50 mM Tris, pH 7.4 at 22°C containing 0.15 M NaCl, 10% glycerol [v/v], 1% NP-40 [v/v], 1 mM NaF, 1 mM Na_3VO_4, 1 mM AEBSF, 1 mM EDTA, 150 μM bestatin, 10 μg/ml E-64, 1 μM leupeptin and 1 μM aprotinin) as previously described [67,68] and used for the following experiment. In brief, an Econo-Column (1.5 x 10 cm, i.d., BIO-RAD) was packed with Protein A-Sepharose (Amersham Biosciences/GE Life Sciences) and washed with PBS pH 7.4 at 22°C at a flow rate of ~20 ml/hour. Anti-adjudin IgG was immobilized onto Sepharose beads by re-circulation and the column was washed extensively to remove unbound IgG. Thereafter, ~500 mg testis lysate was re-circulated for 3–4 hours at room temperature, and the column was washed with ten column volumes of PBS pH 7.4 at 22°C. Bound proteins were eluted with elution buffer (0.2 M glycine, pH 2.5 at 22°C) and designated as adjudin binding proteins. The pH of this eluted sample was reconstituted to 7.4 using Tris, the sample was concentrated using a Microcon centrifugal filter unit (Mr cut-off at 10 kDa) and an aliquot was resolved by SDS-PAGE for Coomassie blue gel staining. Proteins were subjected to in-gel digestion by trypsin and identified by liquid chromatography-mass spectrometry (LC-MS) which was performed at the Proteomics Resource Center, The Rockefeller University. Results obtained from LC-MS experiments were confirmed by SDS-PAGE and immunoblotting, which were performed by standard protocols.[67,68]

Competitive binding assays. Competitive binding assays were performed to validate the pull-down of testin and actin by anti-adjudin IgG. In brief, binding buffer (see above), testis lysate (0 and 4 hours and 4 day post-adjudin treatment), [^{3}H]-adjudin (30 x 10^4 cpm) and anti-actin IgG (1–2 μl; Santa Cruz Biotechnology; cat # sc-7210, lot # G1404 or cat # sc-1616, lot # K0405) or anti-testin IgG (1–2 μl)[22,23] in the absence (to assess total binding) or presence (to assess displacement, thereby monitoring binding specificity) of 5 μg unlabeled adjudin (1 mg/ml stock prepared in ethanol). All samples were combined in a final volume of 500 μl. Samples were vortexed and incubated at 4°C for 36 hours. Thereafter, *Sac* cells were added and samples were incubated at 4°C for 1 hour. Samples were centrifuged and radioactivity quantified in pellets in a β-counter with 1 ml of scintillation fluid. As a control, rabbit IgG was used in

place of anti-actin or anti-testin IgG. Non-specific binding was assessed by using binding buffer containing 0.5% BSA [w/v].

General methods. Protein concentrations were determined by the bicinchoninic acid protein assay (Thermo Scientific). Paraffin embedment of testes and H&E staining were performed using routine protocols. Light microscopy was carried out by using a Nikon Eclipse 90i microscope with a built-in DS-Fi1 12.5 MPx digital camera with Nikon NIS-Elements imaging software package (version 3.2). Statistical analyses were performed by Student's t-test or two-way ANOVA followed by Tukey's post hoc test using InStat software package (version 3.01; GraphPad Software, Inc.). $p < 0.5$ was taken as statistically significant.

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