Spermatogenesis rescue in a mouse deficient for the ubiquitin ligase SCF\(\beta\)-TrCP by single substrate depletion

Naama Kanarek, Elad Horwitz, Inbal Mayan, Michael Leshets, Gady Cojocaru, Matti Davis, Ben-Zion Tsuberi, Eli Pikarsky, Michele Pagano, and Yinon Ben-Neriah

1The Lautenberg Center for Immunology, Jerusalem 91120, Israel; 2Department of Pathology, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel; 3Department of Pathology, New York University Cancer Institute, New York University School of Medicine, New York, New York 10016, USA; 4Howard Hughes Medical Institute, New York University School of Medicine, New York, New York 10016, USA

\(\beta\)-TrCP, the substrate recognition subunit of a Skp1–Cul1–F-box (SCF) ubiquitin ligase, is ubiquitously expressed from two distinct paralogs, targeting many regulatory proteins for proteasomal degradation. We generated inducible \(\beta\)-TrCP hypomorphic mice and found that they are surprisingly healthy, yet have a severe testicular defect. We show that the two \(\beta\)-TrCP paralogs have a nonredundant role in spermatogenesis. The testicular defect is tightly associated with cell adhesion failure within the seminiferous tubules and is fully reversible upon \(\beta\)-TrCP restoration. Remarkably, testicular depletion of a single \(\beta\)-TrCP substrate, Snail1, rescued the adhesion defect and restored spermatogenesis. Our studies highlight an unexpected functional reserve of this central E3, as well as a bottleneck in a specific tissue: a single substrate whose stabilization is incompatible with testicular differentiation.

Keywords: shRNA transgenic mouse; SCF-E3; \(\beta\)-TrCP paralogs; adherens junctions; Snail1; spermatogenesis

Supplemental material is available at http://www.genesdev.org.

Received August 3, 2009; revised version accepted December 24, 2009.
transient, significant, yet incomplete ablation of β-TrCP2, we created a mouse model that overcomes the limitation of traditional conditional ablation studies. Our β-TrCP2 ablation protocol on a β-TrCP1-null background simulates a drug inhibitory effect, and may therefore predict tissue-specific effects and systemic tolerance to β-TrCP-based therapeutic intervention.

Results

Mice deficient in both β-TrCP isoforms have a severe testicular phenotype

The shβ-TrCP2 transgene is induced by tetracycline treatment [Fig. 1A], causing a variable reduction of β-TrCP2 mRNA in different tissues ranging from 60% to 90% (Supplemental Fig. S1C). Mouse embryonic fibroblasts (MEFs) derived from the hybrid strain (shβ-TrCP2 transgenic mice crossed with β-TrCP1 knockout mice, denoted KO1/KD2) had 15%–20% residual β-TrCP2 mRNA levels upon doxycycline treatment. These MEFs had stabilized phosphorylated β-catenin and IκBα levels following TNFα treatment. shβ-TrCP2 (KD2) alone, and particularly in combination with a single allelic loss of β-TrCP1, led to intermediate substrate stabilization, whereas MEFs from β-TrCP1 knockout (KO1) were comparable with wild type (Supplemental Fig. S1B). Surprisingly, there were no signs of illness or malfunction in tetracycline-treated mice observed for many weeks, nor did we detect gross tissue abnormalities in the induced hybrid mice, even at tissues with residual β-TrCP2 expression of 10%—all with the exception of the testis (Fig. 1C, Supplemental Fig. S1C). Four weeks following the initiation of tetracycline treatment, the hybrid testes (KO1/KD2) [n = 32] were much smaller than those of control heterozygous, KO1 [n = 8], or KD2 mice [n = 29] [Fig. 1B]. Whereas the KD2 testes resembled wild-type mice histologically, KO1/KD2 testes showed disordered tissue architecture and lack of mictic cells, spermatids, and sperm with 100% penetrance. The lumen of the KO1/KD2 seminiferous tubules is occupied by cells with homogeneously distributed chromatin [Fig. 1D, top panel; Supplemental Fig. S1D], suggesting that they are dislocated spermatogonial stem cells [Dettin et al. 2003]. We verified the identity of these cells by immunostaining for a mitosis marker [phospho-Histone H3] [Fig. 1D, middle panel] and a specific type A spermatogonial cell marker, Oct3/4 [Fig. 1D, bottom panel; Gidekel et al. 2003]. The ectopic localization of the type A spermatogonial cells and the deformation of the seminiferous tubules could indicate an impairment in cell–cell interaction that is needed for spermatogenesis [Xia et al. 2005a; Oatley and Brinster 2008].

A nonredundant role of the β-TrCP isoforms in the spermatogenesis process

The phenotype observed in KO1/KD2 testes is distinct from the one associated with KO1 alone, as the latter affects metaphase 1 spermatocytes while preserving the normal morphology and localization of type A spermatogonial cells [Guardavaccaro et al. 2003]. To trace the possible cause of the phenotype divergence, we compared the mRNA expression levels of the two β-TrCP isoforms in different testicular cell types [Namekawa et al. 2006] versus a median pan-tissue expression [Lattin et al. 2008]. β-TrCP1 is expressed in spermatogonia at median expression levels (Fig. 1E, blue line and gray dot), yet is highly expressed in meiotic spermatocytes [Fig. 1E, blue line]. Quite the opposite is the expression of β-TrCP2, with median levels in spermatogonial cells, but low expression [20% of the median] in meiotic cells [Fig. 1E, pink line and pink dot]. Accordingly, β-TrCP1 knockout will not affect spermatogonia, only meiotic cells that rely on β-TrCP1 due to their low β-TrCP2 expression. KD2 alone will not have any testicular effect, since β-TrCP1 is preserved at sufficient functional levels [Fig. 1E, green triangle; Kudo et al. 2004] in all cell types. KO1/KD2 will then compromise total β-TrCP expression in spermatogonia to the extent that they cannot differentiate. Based on the distinct testicular phenotypes of β-TrCP1 knockout versus double-isoform β-TrCP ablation, it is tempting to speculate that one reason for the evolutionary conservation of two functional β-TrCP paralogs is in conjunction with regulation of male germ cell differentiation. Thus, β-TrCP1-specific modulation may adjust the pace of germ cell maturation without affecting spermatogonia—the germ lineage stem cells.

The testicular phenotype is reversible and is associated with impaired cell junctions

The testis is a highly dynamic tissue and has the capacity to restore itself once the cause of disorder is removed [Brinster 2002; Mruk et al. 2006]. To study the recovery potential of the KO1/KD2 testes, we took advantage of one unique characteristic of the shβ-TrCP2 transgenic system—its reversibility—and compared the two testes of a KO1/KD2 mouse [n = 5] by harvesting them consecutively [Supplemental Fig. S2]. Following a 4-wk tetracycline treatment, one testis was removed for analysis, tetracycline was discontinued, and, 4 wk later, the other testis was harvested. A nearly full reversion of the phenotype was observed, with repopulation of the seminiferous tubules by a full spectrum of the differentiating cells [Fig. 2, top panel] and relocation of the spermatogonial cells to the periphery of the tubules [Fig. 2, middle and bottom panels].

Spermatogenesis is associated with extensive cell junctions restructuring at the Sertoli–Sertoli cell and Sertoli–germ cell interface [Yan et al. 2007]. Considering the well-known plasticity of the testicular cell junctions and plausible contribution of defective intercellular interactions to the observed phenotype, we studied the different cell junctions in the KO1/KD2 testes. As disruption of the Sertoli–germ cell adherens junctions does not affect the tight junction integrity [Xia et al. 2005b], it was important to distinguish between damaged tight junctions [which maintain the blood–testis barrier] and adherens junctions [which control the trafficking of differentiating cells from...
The protein ZO-1 is localized primarily at tight junctions and in a subset of adherens junctions (the ectoplasmic specialization [ES] junctions between Sertoli and elongated spermatids) (Byers et al. 1991). ZO-1 staining revealed the presence of intact tight junctions in KO1/KD2 testes, whereas adherens junctions were absent (Fig. 3A; Supplemental Fig. S3A). In accord with this, E-cadherin, a key component of adherens junctions, was severely reduced in the KO1/KD2 testes (Fig. 3B). Transmission electron microscopy of wild-type cells demonstrated long and close adhesions between Sertoli cells and neighboring primary spermatocytes with apparent association with cytoskeletal filaments, characteristic of adherens junctions, whereas no such junctions were observed in the KO1/KD2 testis, and their cell-to-cell adhesions were limited to short, intercellular adhesions (Supplemental Fig. S3B). Upon tetracycline omission, both ZO-1 and E-cadherin reverted back to normal localization and expression levels (Fig. 3C), supporting the notion that disruption of the adherens junctions plays a major role in the disordered structure of the KO1/KD2 testes.

Figure 1. A testicular phenotype following inducible β-TrCP2 knockdown on a β-TrCP1 knockout background. (A) The viral construct used for generation of the β-TrCP2 knockdown transgenic mice. H1 promoter and a Tet Operator regulate the shRNA sequence targeting β-TrCP2. The shRNA transcription is inhibited by the Tet Repressor, which is constitutively transcribed under the human EF1α promoter. In the presence of tetracycline, the Tet Repressor is released from the Tet Operator, permitting the shRNA transcription. The Tet Repressor is fused to GFP for easy monitoring of the transgene’s expression. (B) Reduced testes size in mice lacking both β-TrCP1 and β-TrCP2. (KD2) β-TrCP2 knockdown mice treated with 1 mg/L tetracycline in the drinking water for 1 mo. (KO1/KD2) β-TrCP1 knockout combined with β-TrCP2 knockdown. (C) β-TrCP1 and β-TrCP2 mRNA levels in the testes by qPCR. Each bar stands for one representative mouse (n = 5–7). (D) Testis histology in wild-type, KD2, knockout (KO1), and KO1/KD2 mice. (Top panel) H&E staining showing normal seminiferous tubules in wild-type and KD2 testes, the KO1 phenotype described by Guardavacaro et al. (2003), and disorganized seminiferous tubules in the KO1/KD2 testis, with the absence of meiotic cells, spermatids, and sperm, and dislocated cells within the lumen. Bar, 20 μm. (Middle panel) Phospho-Histone H3 staining (red) and Hoechst counterstaining (blue) demonstrating mitotic cells (type A spermatogonia) that are confined to the seminiferous tubules’ periphery in wild-type, KD2, and KO1 testes, but dislocated to the lumen of the KO1/KD2 seminiferous tubules. (Bottom panel) Oct3/4 staining, a marker for type A spermatogonia, indicating the identity of the cells in the lumen of KO1/KD2 seminiferous tubules. Bar, 20 μm. (E) Differential mRNA expression of β-TrCP1 and β-TrCP2 in sperm development, based on data from Namekawa et al. (2006). Median pan-tissue and mammary gland expression values of the two mouse β-TrCP paralogs, based on Lattin et al. (2008), are indicated as a reference to the testicular values. Probe set expression was normalized using the flowing Value = [Value − Mean(Row)]/Standard deviation (Row). The Y-axis value represents the arbitrary normalized expression. Expression data were derived from two biological repetitions, and standard deviation is indicated.
Snail1 depletion rescues a β-TrCP-deficient mouse

E-cadherin levels are transcriptionally controlled by Snail1 (Cano et al. 2000), a bona fide substrate of β-TrCP (Zhou et al. 2004). Snail1 immunostaining and Western blot analyses showed remarkable up-regulation of the protein levels in spermatogonia of the KO1/KD2 mice (Fig. 4A,B, lanes 1,3,5,7). To test whether Snail1 accumulation is responsible for the reduction of E-cadherin and impairment of the adherens junctions [Mruk et al. 2008], we depleted Snail1 in the testis by infecting one KO1/KD2 testis with shRNA lentivirus targeting Snail1, while the other testis was infected with control shRNA. Both testes were harvested together, either 11 or 15 d post-infection [Supplemental Fig. S4]. The depletion of Snail1 was analyzed by Western blot [Fig. 4B] and immunohistochemistry [Fig. 4C,D, middle panel]. Indeed, Snail1 depletion resulted in restoration of E-cadherin expression to nearly normal levels [Fig. 4C,D, bottom panels]. Remarkably, Snail1 knockdown virtually rescued the entire spermatogenesis defect of β-TrCP deficiency, evident by the overall organization of the seminiferous tubules 11 d post-infection (Fig. 4C, top panel) and the recovery of spermatogenesis at 15 d after Snail1 knockdown (Fig. 4D, top panel). We conclude that the spermatogenesis defects following ablation of both β-TrCP isoforms were largely due to Snail1 stabilization, a most likely cause for the reduction of E-cadherin and impairment of adherens junctions. Notably, the spermatogenesis defect observed upon β-TrCP1 knockout only is presumably due to stabilization of a different substrate, Emi1 [Guardavaccaro et al. 2003].

Discussion

The SCF ([Skp1/Cullin/F-box protein] comprises a large subfamily of modular E3s—denoted Cullin Ring ubiquitin Ligase (CRL)—that control ubiquitination of many substrates, typically in a phosphorylation-dependent manner [Willems et al. 2004; Deshaies and Joazeiro 2009]. Only a minority of the SCF E3s has been assigned to established substrates, mostly regulators of the cell cycle and transcription, yet numerous studies have affirmed their important roles in controlling cell size, proliferation, and survival [Fuchs et al. 2004; Nakayama and Nakayama 2006]. Certain SCF E3s, such as SCFSkp2 and SCFβ-TrCP, have been shown to promote tumorigenesis and could be considered as potential targets for anti-cancer therapy [Frescas and Pagano 2008]. To that end, a potent small molecule CRL inhibitor, targeting an essential Cullin-modifying enzyme [Nedd8 activation enzyme [NAE]] has been developed recently and proved valuable in suppressing tumor growth [Soucy et al. 2009]. Nevertheless, it is hard to predict the feasibility of systemic in vivo inhibition of the entire CRL E3 family, or even a single CRL like SCFβ-TrCP, which controls the turnover of multiple essential cellular proteins. We learned that very high-level systemic inhibition of β-TrCP is compatible with the well-being of treated mice, indicating a considerable robustness of physiologic cellular protein turnover. This feature likely depends on the small residual activity of β-TrCP2, rather than other compensatory mechanisms. Our preliminary studies of another tissue (the mouse gut) indicate that, whereas 20% β-TrCP2 expression on a β-TrCP1-null background [Supplemental Fig. S1C] is compatible with normal tissue homeostasis, complete ablation of both β-TrCP isoforms in the same tissue is fatal. Conceivably, cancer cells, in contrast to normal cells, are far more vulnerable to deregulation of protein turnover [Adams 2004]; an example is multiple myeloma cells succumbing to partial intermittent proteasomal inhibition [LeBlanc et al. 2002]. However, our study of the testis indicates that this distinction between normal and malignant cells may not always hold true. If a system relies on dynamic protein turnover, as necessary for continuously remodeling adheres junctions in the renewing testis, deregulating a single key component of this process, like Snail1, may greatly compromise homeostasis [Perez-Moreno et al. 2003]. On the other hand, the testis is an example of a tissue having a remarkable plasticity that is capable of restoring its normal architecture and function shortly after terminating the SCFβ-TrCP inhibition.

Figure 2. Complete reversion of the testicular disorder following withdrawal of the shRNA inducer. (Top panel) H&E staining of the right and left testes of a single KO1/KD2 mouse, showing disorganized seminiferous tubules of the left testis. Four weeks after omitting the tetracycline treatment, the seminiferous tubules of the right testis display normal structure. Bar, 10 μm. [Middle panel] Mitotic cells (stained in red for phospho-Histone H3) are present in the lumen of seminiferous tubules of the KO1/KD2 testis (left), but are found in their normal location at the periphery, following tetracycline withdrawal (right). (Blue) Hoechst staining. (Bottom panel, left) Type A spermatogonial cells (stained by Oct3/4) that were aberrantly positioned in the lumen relocated to their normal position in the right testis of the same mouse.
Should this property characterize other rapidly renewing adult tissues, temporary SCF\(\beta\)-TrCP inhibition may be systemically tolerable, providing means of specifically affecting tumor cells, and could even be harnessed for other medical benefits, such as developing male contraceptives.

Materials and methods

shRNA lentiviral vectors targeting \(\beta\)-TrCP and Snail1

The shRNA lentiviral vector was constructed by inserting an XmaI–NotI fragment from pEGFP-N1 (Clontech) and the hEF1\(\alpha\) promoter into the pcDNA6/TR plasmid (Invitrogen) at the EcoRI and SpeI–HindIII sites, respectively. The hEF1\(\alpha\) promoter-Tet Repressor-GFP cassette was then excised with BglII and inserted into a lentiviral backbone vector (Zufferey et al. 1998) at the PstI and MscI sites. The \(\beta\)-TrCP targeting shRNA cassette was generated using the pTER system (van de Wetering et al. 2003), cut by EcoRI, and inserted to the lentiviral backbone plasmid at the EcoRV site. Plasmids encoding shRNA targeting mouse Snail were purchased from Open BioSystems. shRNA sequences were as follows: \(\beta\)-TrCP2 shRNA, GGACTTGTGCATTAAGTAC; Snail1 shRNA, GCCCAACTATAGCGAGCTGCA.

Lentivirus production

High-titer lentiviral vectors were generated by transfecting 10 14-cm plates of HEK 293T cells with the lentiviral backbone along with the packaging plasmids [gag-pol and pMD.G] using the BBS/calcium phosphate transfection reagent [50 mM BES, 280 mM NaCl, 1.5 mM Na\(_2\)HPO\(_4\) at pH 6.95, 0.25 M CaCl\(_2\)]. Supernatant was centrifuged in an ultracentrifuge [Beckman Coulter Avanti, J-30I]. The pellet was resuspended in 200 \(\mu\)L of KOSOM transgene buffer (Chemicon).

Generation of transgenic mice

Generation of transgenic mice using lentivirus transduction was performed as described [Lois et al. 2002; Singer et al. 2006]. In brief, the concentrated virus was injected into the perivitelline space of C57Bl6/F1 zygotes. Following a 3-d incubation period, the developed blastocytes were checked for expression of GFP using confocal microscopy, and were transferred into pseudo-pregnant foster mothers. Adult founder mice were bred with C57Bl/6 mice (Harlan Laboratories).

Production of MEFs

MEFs were prepared from 12.5-d-post-coitum embryos and seeded onto 10-cm plates precoated with 0.5% gelatin. Culture was maintained in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum [Beit Haemek].

RNA and protein extraction, quantitative PCR (qPCR) analyses

RNA was recovered from tissues using Sigma Tri-Reagent according to the manufacturer’s instructions. cDNA was prepared using...
Snail1 depletion rescues a β-TrCP-deficient mouse

**Figure 4.** Stabilization of Snail1 is responsible for the spermatogenesis defect of β-TrCP-deficient mice. (A) Snail1 staining is restricted to spermatogonia at the periphery of wild-type, KD2, and KO1 seminiferous tubules, but is elevated in both peripheral and centrally dislocated spermatogonia of KO1/KD2 testes. Original magnification degree and scale are indicated. (B, lanes 1,3,5,7) Western blot analysis demonstrating stabilization of Snail1 protein in KO1/KD2 testes in comparison with KO1, KD2, and wild-type testes. The right testis of each mouse was infected by lentiviral control shRNA (ctrl), and the left testis was infected by lentiviral shRNA targeting Snail1 (Snail). (Lanes 1,2) The reduction of Snail1 protein following the in vivo knockdown in the left testis is shown. (Lanes 1,2) Some elevation in β-catenin protein levels is observed in the KO1/KD2 testes. (C) Comparison of two testes of a KO1/KD2 mouse 11 d post-infection. One testis was infected with shCtrl, and the second testis was infected with shSnail1. (Top panel) H&E staining showing disorganized seminiferous tubules in the shCtrl testis (two left panels) and better-organized seminiferous tubules in the shSnail1 testis (two right panels). No centrally dislocated spermatogonia are observed after shSnail1 infection. The original magnification degree and scale are indicated. (Middle panel) Snail1 staining showing stabilized Snail in shCtrl testis and its significant reduction following Snail1 knockdown. (Bottom panel) E-cadherin levels are increased following the knockdown of its transcriptional repressor, Snail1. (Green) E-cadherin, (red) ZO-1. (D) Comparison of two testes of a KO1/KD2 mouse 15 d post-infection. Data are presented as in D. The testis architecture is fully restored following shSnail1 knockdown: Mature sperm is found [black arrows in the top right panel].
MMLV Reverse Transcriptase [Invitrogen]. Quantitative real-time
PCR analysis was performed using the Platinum SYBR green reagent [Invitrogen] and an ABI prism 7900 real-time thermocycler. Protein extracts were prepared in protein lysis buffer [50 mM Tris, 420 mM NaCl, 5 mM EDTA] supplemented with protease and phosphatase inhibitors.

Antibodies and immunohistochemistry
The following antibodies were used: phosphorylated β-catenin (Ser33, Ser37, and Thr41), phosphorylated IκBα (Ser36), β-catenin [Cell Signaling], IκBα [Santa Cruz Biotechnologies], E-cadherin [BD Transduction Laboratories], phospho-Histone H3 [Upstate Biotechnologies], Snail1 [Abcam], ZO-1 [Zymed], Oct3/4 rabbit antiserum [Gidekel et al. 2003]. Immunohistochemical analyses were performed using standard procedures. Antigens were retrieved from either formaldehyde or bouin-fixed paraffin-embedded 5-μm sections in a decloaking chamber [Biocare Medical] in 20 mM Citrate buffer [pH 6]. Antibodies were hybridized in CAS-BLOCK [Zymed]. HRP-conjugated secondary antibodies were goat anti-rabbit [Nichirei Bioscience] and goat anti-mouse [Dako]. Chromogen substrate for HRP reaction was DAB plus [Thermo Scientific]. Fluorescent secondary antibodies were Alexa 488 or Alexa 647 goat Ig conjugates [Invitrogen].

qPCR primers
qPCR primers used were as follows: β-TrCP1 forward, AAAT AACCCAACACTGGCCTCA; β-TrCP1 reverse, TCATCCTGCTGTTGGAGGAGT; β-TrCP2 forward, TGGCGCCTATGATGGTCTCA; β-TrCP2 reverse, GTCAAGAGCAGCCTGCAAGTC.

Animal procedures
All procedures were approved by the Animal Care and Use Committee of the Hebrew University of Jerusalem. Animals were provided food and water ad libitum. Removal of the testis was performed as follows: Mice were anesthetized with Ketamin-Xylasine (200 mg/kg and 10 mg/kg, respectively). The skin around the testis area was incised and one testis was exposed. Removal of the testis was performed by cutting the testis and tying the blood vessels with a polyamide string. Lentiviral transduction of the testis was performed by anesthetizing the mice and injecting 25 μL of concentrated virus into the testis capsule. Following each surgical procedure, wounds were sutured with a polyamide string, and mice were subcutaneously injected with 100 μg of Rimadyl [Pfizer] in 1 mL of sterile PBS.

Transmission electron microscopy
Animals were sacrificed as described and testes were removed and immediately fixed by Karnovsky’s fixative. Following CaCodylate buffer washes and post-fixation with 2% Osmo, samples were dehydrated in ethanol gradient and transferred into propylene oxide. Embedding was done in beam capsules by EMbed 812 Resin [EMS no. 14120] for 48 h at 60°C. Thin sections (50–70 nm) were stained with uranyl acetate and lead citrate and were examined under a Philips EM 12P [voltage, 100 kV] electron microscope.

Acknowledgments
We thank Mina Fogel [Kaplan Medical Center] for assisting with Snail1 analyses, Ezra Rahamim [Hebrew University, Hadassah Medical Centre] for the electron microscopy analysis, and Benny Geiger [Weizmann Institute] and Dov Sofer [Hadassah Medical Center] for their helpful advice on cell adhesion structures through electron microscopy and immunohistochemistry. This work was supported by grants from the RUBICON Network of Excellence, [EU-6th Framework], Dr. Miriam and Sheldon G. Adelson Foundation for Medical research [AMRF], the Israel Science Foundation, Israel Cancer Research Fund [ICRF] USA, National Institutes of Health [R01-GM057587, R37-CA076584, and R21-CA125173], and the Multiple Myeloma Research Foundation to M.P. M.P. is an Investigator with the Howard Hughes Medical Institute.

References


