The Glial Cell-Derived Neurotrophic Factor (GDNF)-responsive Phosphoprotein Landscape Identifies Raptor Phosphorylation Required for Spermatogonial Progenitor Cell Proliferation*

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Cytokine-dependent renewal of stem cells is a fundamental requisite for tissue homeostasis and regeneration. Spermatogonial progenitor cells (SPCs) including stem cells support life-long spermatogenesis and male fertility, but pivotal phosphorylation events that regulate fate decisions in SPCs remain unresolved. Here, we described a quantitative mass-spectrometry-based proteomic and phosphoproteomic analyses of SPCs following sustained stimulation with glial cell-derived neurotrophic factor (GDNF), an extrinsic factor supporting SPC proliferation. Stimulated SPCs contained 3382 identified phosphorylated proteins and 12141 phosphorylation sites. Of them, 325 differentially phosphorylated proteins and 570 phosphorylation sites triggered by GDNF were highly enriched for ERK1/2, GSK3, CDK1, and CDK5 phosphorylation motifs. We validated that inhibition of GDNF/ERK1/2-signaling impaired SPC proliferation and increased G2/M cell cycle arrest. Significantly, we found that proliferation of SPCs requires phosphorylation of the mTORC1 component Raptor at Ser863. Tissue-specific deletion of Raptor in mouse germine cells results in impaired spermatogenesis and progressive loss of spermatogaonia, but in vitro increased phosphorylation of Raptor by raptor over-expression in SPCs induced a more rapidly growth of SPCs in culture. These findings implicate previously undescribed signaling networks in governing fate decision of SPCs, which is essential for the understanding of spermatogenesis and of potential consequences of pathogenic insult for male infertility. Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.065797, 982–997, 2017.

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Mitotic self-renewal of stem cells is essential for tissue homeostasis and regeneration and usually relies on extrinsic stimuli from cytokines that are released by supporting cells within the stem cell niche. In the male gonad, continual self-renewal of spermatogonial stem cells (SSCs)1 ensures the maintenance of the stem cell pool. Mitotic division and initial differentiation of SSCs produces Apaired (Apr) and Aaligned (Aal) type germ cells, which remain connected through intercellular bridges (1). These cells are the spermatogonial progenitor cells (SPCs) of the male testis that give rise to all cells of the spermatogenic lineage and support life-long spermatogenesis (2). Self-renewal and proliferation of mouse SPCs requires glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor beta super family that is secreted from Sertoli cells or peritubular myoid cells of the testis niche (3–6). GDNF is a potent trophic factor that promotes cell survival and proliferation in various organs and is required for the development and maintenance of enteric, sympathetic, and sensory neurons and the renal system (7). In mouse testis, lack of GDNF results in depletion of the stem cell pool because of impaired self-renewal of SSCs, whereas overexpression of GDNF induces accumulation of spermatogonia (3). SPC self-renewal and proliferation is GDNF-dependent in many mammalian species including mice and human (5, 8–10). Known GDNF-responsive regulatory networks overlap between somatic lineages and SPCs, such as RET receptor tyrosine kinase-mediated activation of the transcription factor ETV5, leading to up-regulation of genes essential for kidney branching morphogenesis (11) and SPC self-renewal and proliferation (12–14).

Dynamic protein phosphorylation which results from the opposing actions of kinases and phosphatases, is a common

1 The abbreviations used are: SSCs, spermatogonial stem cells; SPCs, Spermatogonial progenitor cells; GDNF, glial cell-derived neurotrophic factor; MAPK, mitogen-activated protein kinase; SFK, SRC family kinase; mTORC1, mTOR Complex 1; LC, liquid chromatography; MS, mass spectrometry; TiO2, titanium dioxide; FDR, false discovery rate; WT, wild type; OE, overexpressing.
and powerful regulatory mechanism involved in the control of cell growth proliferation, and survival in response to intracellular or extracellular stimuli. In vitro cultured SPCs are heterogeneous with a subpopulation of functional stem cells. Downstream kinases implied GDNF signaling in SPCs include mitogen-activated protein kinase (MAPK), PI3K/AKT, and SRC family kinase (SFK) (15–17). The precise role of these kinases and their associated networks in SPCs remains to be elucidated, and current evidence suggests that SPC proliferation and self-renewal is regulated by the interplay of multiple GDNF-responsive pathways. For instance, an active myristoylated form of Akt-Mer (myr-Akt-Mer) can support proliferation of SPCs in the absence of GDNF (16), implying PI3/AKT in self-renewal. Other data supports a scenario in which PI3K/AKT and SRC kinase mediated signaling play distinct roles for SPC survival and self-renewal, respectively (17). Transgenic expression of an activated form of H-RAS, a potent PI3K/AKT activator, or of cyclin D2, allows for long-term survival and proliferation of SPCs without GDNF supplement in culture (18). However, proliferation of transgenic H-RAS SPCs was also sensitive to MEK/ERK pathway inhibitors, illustrating the complexity of GDNF-induced signaling networks in SSCs (18). SPCs proliferate slowly, with a doubling time of ~4–6 days in vitro (4, 5). Therefore, GDNF signaling likely affects both longer-term acting networks required for self-renewal and proliferation of SPCs as well as more immediate signaling pathways such as those regulating survival that may involve transient phosphorylation.

Monitoring phosphorylation dynamics can identify protein kinase networks in response to stimuli and is therefore crucial for our understanding of how differential phosphorylation participates in translating signals into physiological responses (19). With the recent development of quantitative mass spectrometry-based methods including the combination of liquid chromatography (LC) with mass spectrometry (MS), high-quality analysis of differential phosphorylation for biological events has become possible (20, 21). Here, we combined large-scale phosphoproteomic analyses with proteomic analyses to profile kinase activity and protein phosphorylation in SPCs in response to stimulation with the growth factor GDNF. Enriched kinase signal networks included those associated with ERK1/2, GSK3, CDK1 and CDK5, all of which have putative roles in SPC proliferation. We validated that the kinase ERK1/2-associated signaling network is essential for proliferation of SPCs. Our data also indicate that Ser863 phosphorylation of the mTORC1 complex partner raptor may represent a crucial step for SPC self-renewal and mitotic proliferation. These data provide new insight into the mechanisms that govern fate decision of SPCs and male reproduction.

MATERIALS AND METHODS

Cell Culture—SPC cultures were established from 6- to 8-day-old C57BL/6 (B6) or B6;129S-Gt(Rosa)26Sor/J mice (Jackson Laboratories, Bar Harbor, ME, USA) as described previously (12, 22). Briefly, CD90.2 positive cells were enriched by magnetic cell separation with antibody-conjugated microbeads (Cat#130–049, Miltenyi Biotec, Shanghai, China). Cultures were maintained on mitotically inactivated STO (SIM mouse embryo-derived thioguanine- and ouabain-resistant cells, obtained from Dr Alan Bradley, Cambridge, UK) feeder layers in a serum-free medium (23) supplemented with 20 ng/ml recombinant GDNF (Cat#212-GD, R&D Systems, Shanghai, China), 150 ng/ml GFRA1 (Cat#560-GR, R&D Systems), and 1 ng/ml basic fibroblast growth factor (FGF2; Cat 356060, BD Biosciences). For all protein and RNA experiments, SPCs were gently dislodged and collected using a pipetting method that yielded germ cell preparations of high (95%) purity (24).

Animals—All experiments and procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (ID: 2011082112). Mice carrying a floxed allele of Rptor (RptorloxPloxP, RaploxPloxP) were a generous gift from David M Sabatini at the Massachusetts Institute of Technology, MA (26). Mice with a germline-specific null mutation of rap2 were obtained by intercrossing RaploxPloxP with (Tg(Ddx4-cre)1Dcas (Vasa-cre) mice (Cat#J006954, Jackson Laboratory). Presence of the null allele was confirmed by PCR genotyping for theloxP site and the Cre recombinase transgene with primers as follows:

LoxP site-forward-5’-CTCATCGATGGCTGATGTGCTCAG-3’.

LoxP site-reverse-5’-GATGACGATGATGTGCTCAG-3’.

Cre recombinase-forward-5’-CACCTGACGCGTGGTTAAGCCGCGT-3’.

Cre recombinase-reverse-5’-TTCCCCATCTAAACACACAGCAT-3’.

Protein Digestion and TMT Labeling—Following a thorough wash in PBS, cells were lysed for 1 h in ice-cold protein extraction buffer consisting of 8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2, 1% (v/v) EDTA-free protease inhibitor mixture, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 mM PMSF. Lysates were centrifuged at 40,000 × g for 1 h. Cysteine residues were reduced with 5 mM DTT for 25 min at 56 °C followed by alkylation in 14 mM iodoacetamide for 30 min at room temperature (RT) in the dark. Unreacted iodoacetamide was quenched by incubation with DTT for 15 min. Samples were then diluted to 1.6 M urea with 25 mM Tris-HCl, pH 8.2, and digested overnight at 37 °C with trypsin (Sequencing Grade Modified Trypsin, Promega, Fitchburg, WI) in a 1:200 enzyme-to-substrate ratio. Following digestion, peptide mixtures were acidified by TFA (0.4% v/v) (Sigma-Aldrich, Shanghai, China), desalted using a SepPak 1cc tC18 cartridge (Waters, Milford, MA) and then lyophilized.

For TMT 6-Plex labeling, 750 µg purified peptides from each biological replicate were reconstituted in 200 µl TEAB, and labeled by five vials of TMT 6plex labeling reagent (PN: 90066) according to the manufacturer’s instructions. Three
cell lines of GDNF-starved SPCs (overnight GDNF starvation) were prepared for replicates. For each cell line, proteomics analysis was performed to compare the global changes of proteins and phosphorylation sites between untreated and GDNF treated condition (re-exposure to GDNF for 3 h after overnight starvation). Three biological replicates in the untreated group were labeled with TMT-126, TMT-128, and TMT-130, respectively; whereas three biological replicates in the treated groups were labeled with the TMT-127, TMT-129 and TMT-131, respectively. After TMT labeling, all six samples were pooled, purified using Waters SepPak tC18 cartridges, and subjected to proteome and phosphoproteome analyses. The labeling efficiency over 99% was confirmed by mass spectrometry.

**High-pH Reversed Phase (Hp-RP) Fractionation for Proteome Quantification**—100 µg TMT-labeled peptide mixture was resuspended in 110 µl Buffer A (10 mM ammonium acetate, pH 10) and loaded onto a XBridge™ BEH130 C18 column (2.1 × 150 mm, 3.5 µm; Waters) with the UltiMate® 3000 HPLC system at a flow rate of 200 µl/min. Fractions were collected every minute in a 60 min gradient of 0–7% buffer B (90% ACN/10 mM ammonium acetate, pH 10) for 3 min, 7–42% B for 40 min, 42–70% B for 12 min, followed by 5 min at 70% B, monitored at 214 nm based on the UV-light trace, and were further pooled to 20 fractions using the nonadjacent pooling scheme. The fractions were then dried under vacuum for proteome quantification.

**High-pH Reversed Phase (Hp-RP) Fractionation and Phosphopeptide Enrichment**—For phosphoproteome quantification, 4.3 mg TMT-labeled peptide mixture was resuspended in 500 µl Buffer A (2% ACN/5 mM ammonium formate, pH 10) and loaded onto a XBridge™ BEH300 C18 column (10 × 250 mm, 5 µm; Waters) at a flow rate of 3 ml/min (Agilent 1260 series system). Fractions were collected with a 62 µl gradient of 0–10% buffer B (90% ACN/5 mM ammonium formate, pH 10) for 5 min, 10–27% B for 34 min, 27–31% B for 4 min, 31–39% B for 4 min, 39–60% B for 7 min, followed by 8 min at 60% B, monitored at 214 nm based on the UV-light trace each 30 s, and were further pooled to 10 fractions using the nonadjacent pooling scheme. The 10 fractions were then dried under vacuum prior to phosphopeptide enrichment.

TMT-labeled phosphopeptides were first enriched using consecutive TiO2 and IMAC methods as described previously (21). Briefly, 100 µl IMAC beads were washed with 1 ml of IMAC binding buffer (40% ACN (v/v), 25 mM FA in H2O) by three times, and suspended in IMAC binding buffer as a 50% slurry. Desalted peptide was dissolved in 120 µl of IMAC-binding buffer and transferred to 10 µl of prepared IMAC beads, followed by incubation for 60 min. The supernatant containing unbound peptides was collected, dried in vacuum, and used for subsequent TiO2 enrichment. The resin was washed twice with 120 µl IMAC binding buffer and phosphopeptides were eluted by incubation with 40 µl IMAC elution buffer (50 mM K2HPO4/NH4OH, pH 10.0) twice for 15 min. Eluates were neutralized with 40 µl 10% FA and dried by vacuum centrifugation at room temperature.

For TiO2 enrichment, the unbound peptides from the above IMAC enrichment were dissolved in 200 µl loading buffer (65% ACN/2% TFA/saturated with glutamic acid), and incubated with TiO2 beads (GL Sciences, Tokyo, Japan) at a ratio of 1:4 (Peptides/Beads) by weight. Following washes with 800 µl wash buffer I (65% ACN/0.5% TFA) and wash buffer II (65%ACN/0.1% TFA), bound peptides were eluted once with 200 µl elution buffer I (300 mM NH4OH/50% ACN) and twice with 200 µl elution buffer II (500 mM NH4OH/60% ACN). The eluates were dried, purified on C18 StageTips (27), followed by mass spectrometry analysis.

**LC-MS/MS Analysis**—For MS analyses, peptides were suspended in 0.1% FA and analyzed using a LTQ Orbitrap Velos mass spectrometer (Thermo Finnigan, San Jose, CA) coupled on line to a Proxeon Easy-nLC 1000. Peptides were first loaded onto a trap column (75 µm × 2 cm, Acclaim® PepMap100 C18 column, 3 µm, 100 Å; DIONEX, Sunnyvale, CA) at a flow rate of 10 µl/min, and then transferred to a reverse-phase microcapillary column (75 µm × 25 cm, Acclaim® PepMap RSLC C18 column, 2 µm, 100 Å; DIONEX, Sunnyvale, CA) at a flow rate of 300 nl/min. The HPLC solvent A was 0.1% FA, and the solvent B was 100% ACN, 0.1% FA. A 205-min linear gradient (3% to 8% buffer B for 3 min, 8% to 29% buffer B for 176 min, 29% to 41% buffer B for 15 min, 41% to 100% buffer B for 1 min, 100% buffer B for 10 min) was used for protein identification and quantification. A 240-min linear gradient (3% to 5% buffer B for 5 min, 5% to 30% buffer B for 205 min, 30% to 45% buffer B for 21 min, 45% to 100% buffer B for 1 min, 100% buffer B for 8 min) was used for phosphopeptide identification and quantification.

Peptide analysis was performed on a LTQ Orbitrap Velos in data-dependent acquisition mode. An MS survey scan was obtained for the m/z range 400–1800 at a resolution of 60,000, and a low-energy MS/MS scan of every precursor in the linear ion trap (collision induced dissociation, CID) followed by a higher energy MS/MS scan in the octopole collision cell (higher energy collision dissociation, HCD) was acquired from the survey scan for the eight most intense ions (as determined by X-calibur mass spectrometer software in real time). Dynamic mass exclusion windows of 60 s were used, and siloxane (m/z 445.120025) was used as a lock mass.

**Identification and Quantification of Proteins and Sites**—Raw files were searched against the mouse protein sequences obtained from the Universal Protein Resource (UniProt) database (release 2013.07; 73938 entries) (28) using MaxQuant software (version 1.3.0.5) (29). False discovery rates (FDR) were estimated using the target-decoy strategy, and FDR cut-offs were set to 0.01 for sites, peptides, and proteins. Enzyme specificity was full cleavage by trypsin, and two maximum missed cleavage sites were permitted. The minimum required peptide length was set to 6 residues. Carbamidomethyl (C) was set as fixed modification. Variable modificatio-
tions included oxidation (M) and acetylation (protein N-term). For the identification of phosphorylation sites, phosphorylation (STY) was additionally set as variable modification. The mass tolerance for precursor ions was set to 20 ppm at the first search as applied in Maxquant for initial mass recalibration. For the main search, the mass tolerance for precursor ions was set to 6 ppm. The mass tolerance for fragment ions was set to 0.5 Da. The minimum score for phosphorylation sites was 30. We further classified phosphorylation sites into two groups: specific sites (the position and amino acid of the modified site could be identified unambiguously) and ambiguous sites (the corresponding peptide was identified as being modified; however, the position and amino acid of the modified site could not be determined because of lack of specific fragment ions). Functional annotation was conducted for both specific and ambiguous phosphorylation sites, prediction of regulatory kinases was performed only for specific sites. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (30) partner repository with the data set identifier PXD005756.

Protein quantification was based on the reporter ion intensities of TMT6 reagents at the peptide level (31). Relative expression values for each protein were calculated by combining MaxQuant identification results with a local modified Libra algorithm for extracting reporter ions (32, 33). In brief, each channel of reporter ion intensity was normalized by the sum of the signals in the corresponding channels. For each peptide, spectra with intensities that deviated from the mean by more than 2-fold of sigma were removed. Each peptide channel was then re-normalized by the sum across channels. The protein intensity was calculated as the median of normalized intensities of the corresponding peptides. For the quantification of phosphorylated sites, expression levels were additionally normalized according to the calibration ratios of total protein abundances. Whole proteome and phosphoproteome results are provided in supplemental Table S1 and supplemental Table S2, respectively. The representative annotated spectra for each phosphorylated site are provided in supplemental Table S2.

Experimental Design and Statistical Rationale—The quantitative proteomes and phosphoproteomes were performed for SPCs after overnight GDNF starvation and re-exposure to GDNF for 3 h, using 3 independent lines as biological replicates (Fig. 1A). Thus, three paired biological replicates were used for proteomic analysis in this study. For statistical comparison of the quantification values in the above proteomics analysis, mean values of protein expression values for each protein in each group were calculated, and compared using the paired Student’s t test between groups. Because isobaric multiplexed quantitative proteomic analyses typically result in an underestimate of fold changes (34), we used GDNF family receptor alpha-1 (Gfra1) as a reference marker for differential expression. Gfra1 was 1.29-fold increased between the 0 h group and the 3 h group ($p < 0.01$). Thus, for the identification of differentially expressed (DE) proteins, the cut-offs for the fold change and $p$ value were set to 1.2 and 0.05, respectively. The Student’s t test with or without a fold change cutoff strategy has been applied for the identification of DE proteins based on quantitative proteomics in many other studies (35–37). The same cut-offs were applied for the identification of differentially regulated phosphorylation sites. However, to obtain confident results, we removed differentially expressed sites if the corresponding protein expression levels were also significantly changed with the same trend (i.e. expression changes would reflect the change in total protein abundance).

Bioinformatics Analysis—To obtain an overview of the function of proteins identified by our proteomic analyses, we applied Gene Ontology (GO) and pathway (KEGG and Wiki-Pathway) enrichment analyses (25). GO categories contain a group of genes with the same location or function, whereas pathway maps show the association of a group of genes with a specific biological process. Target proteins were mapped to Entrez gene identities for functional annotation, and WebGeneStalt was used for enrichment annotation (38). The whole mouse genome was set as background and an adjusted $p$ value (according to the Benjamini and Hochberg method) less than 0.05 was controlled for significant enrichment. WebLogo 3.4 was used to generate frequency plots of amino acids surrounding modified sites (39). To additionally identify relevant regulatory kinases and phosphatases, we manually searched the phosphorylation relations between kinases and DE sites based on the motifs obtained from the Human Protein Reference Database (HPRD) (40). We further performed enrichment analysis using Fisher’s exact test to find dominant regulatory motifs. A binding motif with larger percentage of phosphorylation sites in DE sites compared with all quantified sites and a $p$ value less than 0.05 was considered statistically significant. The protein-protein interaction network for proteins with up-regulated phosphorylation sites was constructed using the STRING database (version 9.1) with a high confidence score cutoff of 0.7 (41).

qRT-PCR—Total RNA was isolated from cultured SPCs using Trizol (Cat#15596026, Invitrogen, Shanghai, China) according to the manufacturer’s instructions. Following first-strand cDNA synthesis using PrimeScript™ RT Master Mix, PCR was performed using SYBR green master mix (Cat#Q141, Vazyme, Nanjing, China) and StepOne Plus (Applied Biosystems, Shanghai, China). See supplemental Materials for primer sequences.

Lentivirus Transfection—Full-length cDNA sequences encoding Cre recombinase and mouse GDNF were amplified by PCR (PrimeSTAR® GXL DNA Polymerase; Cat#R050A, Takara, Dalian, China) using genomic DNA from Vasa-Cre mice (Stock No:006954; Jackson Laboratories) as a template, and were cloned into the lentivirus vector pCDH-EF1-MCS-T2A-Puro (System Biosciences, Cat# CD500) using EcoR1 (Cat#R0101V, NEB, Beverly, MA) and Not1 (Cat#R0189V, NEB) or Xba1 (Cat# R0145V, NEB) and Bamh1 (Cat# R0136V, NEB) or Xba1 (Cat# R0145V, NEB) and Bamh1 (Cat# R0136V, NEB) and Bamh1 (Cat# R0136V, NEB)
NEB) restriction sites, respectively. PCR primers were forward-5’-GGAATTCCGACCATGCTCAATTTACTGACCGTAC-3’ (adding an EcoR1 site) and reverse-5’-ATGAAGATCGGCGCCATCGCATCCATCTCGACGAGC-3’ (adding a Not1 site) for Cre, and forward-5’-GCTCTAGAGCCACCATGAACTATGGGATGTC-3’ (adding an Xba1 site) and reverse-5’-CGGGATCCGATACATCCACACCGTATAGG-3’ (adding a BamH1 site) for GDNF.

Plasmids (pCDH-CRE or pCDH-GDNF) and packaging plasmids pmd-REV and pmd-1G/pmd-L were cotransfected into HEK293T cells using Lipofectamine 2000 (Cat#11668030, Invitrogen) according to manufacturer’s instructions, and supernatant containing virus was harvested 48 h post-transfection. For viral transduction of SPCs, 200,000 to 300,000 cells were cultured in 1 ml of a 1:1 mixture of culture medium and viral supernatant containing virus was harvested 48 h post-transfection. For viral transduction of testis tissue, mycinc C-treated STO feeder layers in fresh culture medium. After 8 h, cells were collected and replated on mitomycin C-treated STO feeder layers in fresh culture medium. For lentiviral transduction of testis tissue in vivo, GDNF lentivirus particles were concentrated 100-fold by ultracentrifugation at 20,000 rpm for 90 min at 4 °C (Optima L-100 XP Ultracentrifuge), and 10 μl concentrated lentivirus medium were injected into the rete of each testis of 6-week-old C57BL/6 (B6) mice.

Overexpression and Point Mutation of Raptor—The following oligonucleotides were used to create the overexpression (normal Ser863), the phosphorylation-dead phosphorylation (Ser863-Ala) by substitution of Ser to Ala) and the sustained phosphorylation (Ser863-Asp) by substitution of Ser to Asp) in mouse raptor using the above lentivirus-transfection methods and lentivirus vector pCDH-EF1-MCS-T2A-Puro: normal Ser863, forward: 5’-GCCGAGCGCGCCCAACCAACAGG-3’, reverse: 5’-CTTGTGTTTGGCTGGCTGGGC-3’; Ser863-Ala, forward: 5’-GCCGACCGCGCCCAACCAACCAAGG-3’, reverse: 5’-CTTGTGTTTGGCTGGCTGGGC-3’; Ser863-Asp, forward: 5’-GCCCGAGCGCGCCCAACCAACCAAGG-3’, reverse: 5’-CTTGTGTTTGGCTGGCTGGGC-3’; and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’, and the full-length cDNA was created by following primers: forward, 5’-GCCGAGCGCGCCCAACCAACCAAGG-3’, reverse-5’-CTTGTGTTTGGCTGGCTGGGC-3’. Total raptor and phosphorylated raptor Ser863 were then validated by Western blot for each line of SPCs after virus transfection.

Western Blotting—Cultured germ cells were washed with PBS and lysed in RIPA buffer (Cat#P0013C, Beyotime). After removal of debris by centrifugation, lysates were boiled at 95 °C for 5 min in loading buffer (Cat#P0015, Beyotime) for degeneration, separated on 5%–12% Bis-Tris gels and transferred to PVDF blots (Cat#162–0177, BioRad). Membranes were blocked with 5% skim milk for 1 h before incubation with antibodies. Primary antibodies were phospho-Raptor (Ser 863) (Cat#sc130214, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Raptor (Cat#2280S, Cell Signaling Technology), phospho-S6 Ribosomal Protein (Ser240/244) (Cat#5364, Cell Signaling Technology, Shanghai, China), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cat#9106, Cell Signaling Technology), ZBTB16 (Cat#AF2944, R&D Systems), β-Tubulin (Cat#2146, Cell Signaling Technology). Secondary antibodies (goat anti-rabbit IgG/HRP, rabbit anti-goat IgG/HRP and goat anti-Mouse IgG/HRP) were purchased from Zhongshan Biotechnology Co, Beijing, China.

Cell Cycle and Apoptosis Analysis—Cultured germ cells were trypsinized and fixed with 75% ethanol at 4 °C overnight. Fixed cells were washed with ice-cold PBS and incubated with BD Pharamingen™ PI/RNase staining buffer (Cat#550825, BD) for 15 min before flow cytometry with a BD FACSVERS. For flow cytometric detection of apoptotic cells, dissociated cells were rinsed with PBS and stained using FITC Annexin V and propidium iodide (PI) dye solutions (Cat#556547, BD Pharamingen, Shanghai, China) according to the manufacturer’s instructions.

Immunohistochemistry—Mouse testes were fixed with Harvard solution (Lot#108k1314v, Sigma) overnight and embedded in paraffin (Lot#140808, Leica, Beijing, China) for sectioning at 5 μm thickness. Immunostaining of sections was performed for ZBTB16 (Cat#AF2944, R&D Systems) (42, 43), LIN28A (Cat#ab4602, Abcam, Shanghai, China) (44) phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cat#9106, Cell Signaling Technology), and phospho-S6 Ribosomal Protein (Ser240/244) (Cat#5364, Cell Signaling Technology).

RESULTS

Quantitative Proteomic and Phosphoproteomic Profiling of SPC Following GDNF Stimulation—To profile global protein expression changes and phosphorylation in cells in response to GDNF, we first established cell cultures using validated protocols for the long-term in vitro propagation of mouse SPCs (23) (supplemental Fig. S1), we next compared the proteomes and phosphoproteomes of SPCs after overnight GDNF starvation and re-exposure to GDNF for 3 h, using 3 independent lines as biological replicates. After digestion of extracted proteins with trypsin, purified Tandem Mass Tag (TMT)–labeled peptides from untreated (GDNF starved) and treated (GDNF replenished) replicates were pooled, and phosphopeptides were enriched with IMAC (immobilized metal affinity chromatography) followed by titanium dioxide (TiO2) beads (21) (Fig. 1A). All samples were analyzed using LC-MS/MS mass spectrometry, and raw spectra were processed using MaxQuant.

Using a false discovery rate (FDR) cut-off of 0.01 for peptides and proteins, we identified 7921 proteins in SPCs with high confidence (Fig. 1B). Among these, 34 proteins were up-regulated and 22 proteins were downregulated in response to GDNF. These presumptive GDNF-regulated proteins included GFRA1 and CCND2 (supplemental Table S1), which are known factors associated with SSC self-renewal. This regulation was further validated by Western blotting ex-
experiments, confirming the validity of our experimental approach (supplemental Fig. S2). We identified 3382 proteins that were phosphorylated at one or multiple residues, including a total of 12141 phosphorylation sites (supplemental Table S2), which encompassed 10450 serines (86%), 1524 threonines (12.6%) and 167 tyrosines (1.4%) (Fig. 1C). Of the 12141 sites, a total of 4429 sites were identified by IMAC, and 10458 sites were identified by TiO2 (supplemental Fig. S3).

Five hundred seventy (4.7%) sites exhibited significant changes in response to GDNF exposure: phosphorylation of 145 proteins containing 248 sites was upregulated, and phosphorylation of 194 proteins containing 322 sites was downregulated (Fig. 1B and volcano plots in supplemental Fig. S4). Both up- and downregulation of phosphorylation was observed for 13 phosphorylated proteins (Fig. 1B and supplemental Table S3). The degenerated surrounding sequences around the modified sites (six amino acid residues at both termini) are usually used for comparison of sites between different data sets (45). We mapped the 12141 phosphorylated sites to 12087 unique surrounding sequences (Fig. 1D). We further compared the number of sites among our data set, dbPTM, and PhosphoSitePlus databases based on surrounding sequences. F, Sample clustering of sites exhibiting differential levels and patterns of phosphorylation after GDNF depletion and following replenishment; three independent samples were used in each group.

Figure 1. Quantification of the GDNF-regulated proteome and phosphoproteome in SPCs. A, Experimental workflow. Three independent lines were used as biological replicates, and for each cell line, LC-MS/MS was performed to compare global changes in protein expression and phosphorylation levels after GDNF depletion and re-exposure to GDNF. B, Summary of proteome and phosphoproteome analyses. C, Serine, threonine, and tyrosine sites of the phosphoproteome. D, Proportions of amino acid residues surrounding phosphorylation sites. E, Overlaps of phosphorylation sites among our data set (SPC), dbPTM, and PhosphoSitePlus databases based on surrounding sequences. F, Sample clustering of sites exhibiting differential levels and patterns of phosphorylation after GDNF depletion and following replenishment; three independent samples were used in each group.
These data reveal that the mouse SPC proteome contains multiple phosphorylation sites that are subject to distinct and differential phosphorylation in response to presence or absence of GDNF (Fig. 1F). To our knowledge, these phosphoproteomics data sets represent the first reported to date for mouse germline stem cells.

**Signaling Pathways Activated in Response to GDNF Are Enriched for Proteins Involved in SPC Self-renewal and Proliferation**—To gain an overview of the potential function of the 325 proteins that undergo phosphorylation changes upon GDNF treatment, we first classified these proteins according to GO terms (including biological process, subcellular localization, and molecular function) and performed KEGG enrichment analyses (25). Enriched functional terms included cell cycle (41 genes) and related functions, including protein binding (123 genes), nucleic acid binding (66 genes) and kinase activity (16 genes) (Fig. 2A and supplemental Table S3).

The surrounding sequence context, or motif, of a phosphorylation site constitutes the binding site for specific kinases. Screening for known and empirical phosphorylating motifs can be thus be used to predict potential interactions of phosphorylated proteins with regulatory kinases. We identified potential kinase phosphorylating motifs in our dataset using human kinase phosphorylating motifs (40), for which kinase-potential kinase phosphorylating motifs in our dataset using supplemental Table S3. A total of 9637 phosphorylated sites were predicted to be regulated by 136 kinase phosphorylating motifs. Next, we found that a total of 11 and 15 regulatory kinase phosphorylating motifs were enriched among sites with increased and decreased phosphorylation levels in response to GDNF, respectively. Among these, motifs that predict interaction with ERK1/2 kinase were the most frequent (Fig. 2B, and listed in supplemental Table S4, whereas the global kinase phosphorylating motifs among differential phosphorylation sites were listed in supplemental Table S5). To further identify potential interaction partners of proteins with increased phosphorylation levels in response to GDNF, we also evaluated known and predicted protein–protein interaction networks using the STRING database (41). In total, we identified 48 putative interaction partners for 39 proteins from our data set, and 26 of these connections were predicted based on experimental evidence (supplemental Table S6). Among these were components of the mTOR complex, which is an important regulatory pathway controlling cell proliferation (47, 48). Specifically, predicted interactions in our dataset included those of the mTOR Complex 1 (mTORC1) subunit raptor with RPS6 kinase and with EIF4B (Fig. 2C), which would regulate ribosome biogenesis and cap-dependent translation.

**Erk1/2 Kinase Signaling Is Essential for GDNF-stimulated Proliferation of Mouse SPCs**—Changes in phosphorylation in response to GDNF availability occurred most frequently at sites within motifs predicted to be recognized by the ERK1/2 kinase (Fig. 2B). To functionally validate this observation, we inhibited ERK1/2 in cells by exposing cultures to U0126 (49), a high-affinity inhibitor of the MER/ERK kinase. Culture of SPCs in the presence of increasing concentrations of U0126 (5, 10, and 20 μM) was associated with an expected concentration-dependent reduction in the level of phosphorylated ERK1/2 kinase (Fig. 3A) and produced marked changes in SPC morphology after 5 days of culture, with loss of proliferating cell clumps (Fig. 3B). In the presence of 5 μM U0126, small aggregates of 2 or 3 cells were observed in culture wells, whereas cultures exposed 10 and 20 μM inhibitor lacked cell clumps and contained only single cells. Consistent with these observations, treatment with 5, 10, and 20 μM U0126 resulted in a significant reduction of the total number of cells to 50.92%, 22.51%, and 13.65% of cultures exposed vehicle control (DMSO, Fig. 3C). We observed increased apoptosis in SPCs treated with the highest dose of U0126 (20 μM, Fig. 3D). Exposure of cells to lower concentrations (5 and 10 μM) of ERK1/2 inhibitor increased the proportion of cells in G0/G1 phase and decreased the percentage of cells in S phase (Fig. 3E). Furthermore, exposure to the inhibitor at any of the tested concentrations caused G2/M arrest in a significant proportion of SPCs (Fig. 3E), indicating that disruption of Erk1/2 activity inhibits cell division and reduces cell proliferation. We also found that genes associated with self-renewal such as Etv5, Bcl6b, Ccnd2 were downregulated after ERK1/2 kinase inhibition (supplemental Fig. S5).

To link these findings to SPC function, we performed transplantation experiments into germ cell depleted testes. Because exposure to high-dose inhibitor for 24 h appeared to induce irreversible apoptosis or death, we only transplanted untreated and 5 μM ERK1/2 inhibitor treated SPCs. We found that spermatogenic colonies generated by cultured cells treated with 5 μM U0126 showed no statistic difference when compared with controls (Fig. 3F, p = 0.11). These data suggest that stem cell content in SPCs retain intact after a brief period of Erk inhibition (24 h treatment without increased of apoptosis or cell death).

**GDNF/ERK1/2 Signaling Promotes Phosphorylation of the mTORC1-associated Protein Raptor At Ser863**—We identified putative GDNF-responsive signaling pathways in SPCs (Supplemental Table S3, sheet 2). The most significantly enriched pathways were the mTOR pathway (13.1 fold enriched), neurotrophin signaling (7.1-fold), insulin signaling (6.8-folds), mitogen-activated protein kinase (MAPK) signaling (4.3-fold), and the NOD-like receptor pathway (8.1-fold) (supplemental Table S5). Of note, we found that GDNF exposure resulted in phosphorylation of raptor at multiple sites. This protein is a known regulatory component of the mTORC1 complex in regulating mTOR activities (48). Phosphoproteomic data from cultured cells indicate that raptor is phosphorylated at 4 sites (Ser722, Thr853, Ser859 and Ser863, Fig. 4A and supplemental Fig. S6), with the highest level of phosphorylation at Serine863.
Phosphorylation of raptor Ser863 has been identified in several large-scale MS studies (50–52) and constitutes a master biochemical switch that modulates hierarchical phosphorylation of raptor at other residues (50). To assess the functional correlation between raptor Ser863 phosphorylation, GDNF-signaling and ERK1/2 and mTOR pathway activity in SPCs, functional annotations of differentially phosphorylated proteins and sites are presented. Figure 2A shows representative enriched GO terms of proteins with differentially phosphorylated sites. Blue bars represent the gene count (corresponding axis shown on top in blue), and purple data points mark the corresponding p values (-LOG10 transformed, corresponding axis shown at bottom in purple). B, The most significantly enriched (sorted by p value) phosphorylating motifs (and corresponding kinases) predicted for sites with up-regulated phosphorylation in response to GDNF. Blue bars represent phosphorylation site count (corresponding axis shown on top in blue) and purple data points mark the p value (-LOG10 transformed, corresponding axis shown at bottom in purple). C, STRING database prediction of interactions among proteins with significant up-regulation of phosphorylation in response to GDNF. Predicted relations among proteins are based on evidence for co-expression, co-occurrence, experimental, fusion, homology, knowledge, neighborhood, and text mining as marked by line color. Among the relations, the official gene symbol “Rptor” represents Raptor.

Phosphorylation of raptor Ser863 has been identified in several large-scale MS studies (50–52) and constitutes a master biochemical switch that modulates hierarchical phosphorylation of raptor at other residues (50). To assess the functional correlation between raptor Ser863 phosphorylation, GDNF-signaling and ERK1/2 and mTOR pathway activity in SPCs,
**Fig. 3.** ERK1/2 kinase is required for spermatogonial progenitor cell proliferation. 

A, Inhibition of ERK1/2 with U0126 was validated by Western blot. B, In the presence of U0126 (5–20 μM for 5 days) SPC cultures ceased to form typical grape-like colonies (DMSO, vehicle control) and produced only small clusters or single germ cells. C, U0126 significantly impaired SPC proliferation. Cell counts were measured before and after 5 days of culture in the presence of U0126 (5–20 μM). D, Percentage of apoptotic cells after treatment of SPCs with U0126 for 24 h. E, Inhibition of ERK1/2 kinase in SPCs by exposure to U0126 for 24 h significantly decreased the proportion of cells in S phase and caused arrest in G2/M phase. F, SPCs exposed to 5 μM U0126 for 24 h formed spermatogenic colonies after transplantation into germ cell-depleted testes. Cells were washed and transplanted immediately after exposure to U0126.
we evaluated the effects of GDNF deprivation and inhibition of the ERK1/2 and mTOR signaling pathways on protein phosphorylation. In response to GDNF depletion and replenishment, raptor phosphorylation, ERK1/2 kinase as well as mTORC1 signaling (reflected by phosphorylation of RPS6) underwent changes in cultured SPCs (Fig. 4B). Specifically, we observed that phosphorylation level of raptor Ser863 gradually increased following re-exposure to GDNF (10 min up to 3 h), in parallel with increased phosphorylation of RPS6 (reflecting mTORC1 activity). However, U0126-mediated ERK1/2 kinase inhibition caused an apparent reduction of raptor Ser863 phosphorylation and down-regulation of mTORC1 signaling (Fig. 4C). We also found that both ERK1/2 kinase activity and phosphorylation levels of Raptor Ser863 were not affected by 3 h treatment of rapamycin, the inhibitor of mTOR (Fig. 4D), suggesting that raptor Ser863 phosphorylation in response to GDNF treatment might be an upstream signal in regulation of mTORC1 activity.

**mTORC1 Activity and Raptor Phosphorylation Are Associated with GDNF-induced Proliferation of SPC In Vivo**—To validate our phosphoproteome data suggesting that GDNF signaling activates the ERK1/2 kinase and downstream mTORC1 pathways in SPCs, we additionally observed phosphorylation in vivo by overexpression of GDNF in testis somatic cells, which is known to stimulate extensive proliferation of undifferentiated spermatogonia including SSCs in the mouse testis (3).

GDNF overexpressing testes contained large clumps of aggregated germ cells within the center and periphery of the seminiferous tubules (Fig. 5A, supplemental Fig. S7) as expected. Staining for the spermatogonia progenitor marker ZBTB16 confirmed that these cells were accumulating spermatogonia (Fig. 5A). In the normal adult mouse testis, phosphorylated ERK1/2 and RPS6 are restricted to Sertoli cells and spermatogonia, respectively (Fig. 5A). In contrast, the aggregated spermatogonia in GDNF overexpressing testes contained high levels of phosphorylated ERK1/2 (Fig. 5A) and phosphorylated RPS6, which reflects mTORC1 activity (Fig. 5A). Western blotting analysis of protein lysates from wild type (WT) and GDNF overexpressing (OE) testis tissue also indicated increased phosphorylation levels of ERK1/2 and RPS6 in GDNF overexpressing testis, and also increased phosphorylation of raptor Ser863 (Fig. 5B). Because spermatogonia progenitor cells are the only increased population in testes, overexpression of GDNF is sufficient for phosphorylation of ERK1/2 and activates mTORC1 signaling in vivo. These results further validate our phosphoproteome data and suggest that ERK1/2 kinase driven phosphorylation of Raptor Ser863 is required for mouse SPC proliferation.
Genetic Loss of Raptor Results in Loss of Spermatogonia in Mouse Testes—We next determined if the mTOR complex component raptor had an essential function in SPCs. We generated mice with a germline-specific deletion of Raptor by intercrossing mice with a conditional floxed Rptor allele (Rptor<sup>tm1.1Dmsa</sup>, referred to as Rap<sup>loxP</sup>) (26) with mice expressing Cre recombinase under the control of the germ cell-specific VASA promoter (Tg(Ddx4-cre)1Dcas, referred to as Vasa-cre). The testes from 3-week-old males with germ cell-specific loss of Raptor (referred to as GC-Rap<sup>KO</sup>) were notably smaller than those from wild-type mice (Fig. 6A, left panel). Histology revealed severe impairment of spermatogenesis in the testes of GC-Rap<sup>KO</sup> males, with absence of germ cells and spermatogenic epithelium (Fig. 6A, center panel). Immunostaining confirmed the absence of LIN28A positive spermatogonial progenitor cells in testis tubules from 6-week-old-mice, whereas very few LIN28A positive progenitors were found in tubules from 3-week-old-mice (Fig. 6A, right panels). These observations suggest a progressive loss of spermatogonial progenitor cells in the absence of raptor. However, because of the early onset of Vasa-Cre activity in primordial germ cells, germ cell loss may have initiated at early fetal stages. To specifically assess the effects of Raptor deletion in postnatal germ cells, we established an SPC culture line from 6-day-old Rap<sup>loxP</sup> pups and used lentivirus-mediated Cre recombination to produce Raptor-null SPCs. The resulting knockdown of raptor in SPCs was associated with a significant reduction in cell proliferation (Fig. 6B).

Overexpression of Raptor Induced a Faster Growth for In Vitro Cultured SPCs—We next wonder whether overexpression of raptor and sustained phosphorylation of the raptor at ser863 site will alter cell growth. We established three lines of SPCs with different raptor, one line of SPCs with overexpression of total raptor by transfection of raptor into cultured cells (Normal-Ser863), a line of SPCs with a phosphorylation-dead point mutation through substitution of Ser863 by Ala, to compete with the endogenous phosphorylation of Ser863 (Ser863-Site mut-Ala), and a line of SPCs with a point mutation through substitution of Ser863 by Asp, to mimic the phosphorylation of Ser863 (Ser863-Site mut-Asp, Fig. 7A). In contrast to the vehicle virus transfected cells (Control), Total raptor and phosphorylated raptor were all increased in three lines (Fig. 7B); whereas cells with persistent phosphorylation of the raptor (Ser863-Asp) and overexpression of total raptor (Normal Ser863) showed significantly faster growing than phosphorylation-dead point mutation (Ser863-Site mut-Ala) and control culture for the SPCs, ~18–24 days after virus transfection (Fig. 7C).

Collectively, our data reveal that GDNF stimulation of SPCs leads to activation of the ERK1/2 kinase, leading to the phosphorylation of downstream substrates, including multiple sites of the mTORC1 complex interacting partner raptor. This signaling cascade may represent a key molecular mechanism regulating GDNF-dependent SPC self-renewal and proliferation (Fig. 7D).

DISCUSSION

Protein phosphorylation is a ubiquitous post-translational modification affecting most signal transduction processes, and quantitative assessment of phosphorylation dynamics is essential for a comprehensive understanding of cellular be-
Here, we evaluated protein phosphorylation events in SPCs response to GDNF, a growth factor that is essential for their self-renewal and proliferation. In response to exogenous factors, phosphorylation may occur in multiple scenarios defined in other studies reflecting rapid changes and progressive or sustained phosphorylation events after stimulation (54, 55). Consistent with a distinct temporal regulation of different intracellular phosphorylation events in response to a stimulus, our study did not detect differential PI3K/AKT kinase activity after sustained stimulation of SPCs with GDNF. Activation of this pathway, which has been previously shown to be involved in SPC self-renewal, is an immediate change likely within 30 min in response to GDNF stimulation (16, 17).

We found that exposure of SPCs to GDNF for 3 h following an overnight starvation produce changes in the expression level of only 0.7% (56/7921) of all identified proteins, indicating the importance of post-translational regulations. Indeed, much broader changes were found in respect to global (9.6%) and site-specific (4.7%) protein phosphorylation. Analyses of phosphorylated sites and surrounding sequences allowed for the identification of several protein kinase pathways that may represent crucial steps for SPC self-renewal and proliferation (Fig. 2B and supplemental Table S4). Pathways enriched in our study have been previously linked to mitosis and proliferation, such as the “growth-associated” or “M phase-specific” histone kinase (Cdk1), which is essential for the cell cycle and executes all events that are required to drive cell division (56). Proteins undergoing phosphorylation changes in SPCs in response to GDNF also included glycogen synthase kinase 3 (GSK3), which controls cell proliferation in somatic cells and is an important regulator of self-renewal of embryonic stem cells (57, 58), and cyclin-dependent kinase 5 (Cdk5), which may affect cell cycle progression by regulating STAT3 (59), a transcription factor previously found to control cell fate decisions in undifferentiated SPCs (60). Our data also provide insight into site-specific phosphorylation of protein substrates known to be subject to regulation via differential phosphorylation. Among these was FOXO1, a transcription factor required for mouse SPC maintenance (61). FOXO1 localizes to the nucleus and activates gene transcription required for cell proliferation, whereas phosphorylation of FOXO1, particularly at Thr24, Ser253, and Ser316 leads to its translocation from the nucleus to the cytoplasm and subsequent inactivation (62). We detected phosphorylation of FOXO1 at Ser284, Ser253, and Ser316, and found down-regulation at these 3 sites upon GDNF stimulation (supplemental Table S2), implying differential phosphorylation at these sites in the control of SPC pro-

Fig. 6. Genetic loss of Raptor results in severe loss of spermatogonial progenitors in the mouse testis. A, Testis morphology, immunostaining and numbers of LIN28A positive cells in the seminiferous tubules of GC-RapKo and wild-type mice (scale bar, 50 μm). B, SPCs established from RaploxP mice were transduced in vitro with Cre encoding lentivirus, resulting in Rptor deletion. Rptor deletion was associated with a significant reduction in the number of SPCs after 5 days in culture.
**Fig. 7. Overexpression of Raptor in cultured SPCs.**

A. Phospho probabilities represented the Raptor serine863 site of Raptor protein. The following three oligonucleotides were used to create cells with raptor overexpression (normal-Ser863), Ser863 site mutation (Ser863-Ala, Ser replaced by Ala for a phosphorylation-dead site mutation and Ser863-Asp, Ser replaced by Asp for an active mutation). Red-color marked amino acid site and base codons indicated the site of Raptor Serine 863 and the sequence substitution.

B. The overexpression of Raptor was verified through Western blots in SPCs by lentivirus-mediated transfection.

C. A 24-day growth comparison was conducted after virus transfection for SPCs (three replicates were calculated in each lines of cells with mutation or overexpression and error bars indicate Means ± S.D.).

D. A model of GDNF-dependent regulation of SPC proliferation was proposed. In this model, GDNF is secreted from the testis niche and essential for maintaining the male germline progenitor pool. Binding of GDNF to its receptor GFRA1 and the coreceptor RET activates intracellular protein kinase signaling including the upstream effectors Src and Ras, which in turn activate downstream pathways that regulate the expression of genes including Etv5, Bcl6b, and Ccnd2, which are required for SPC self-renewal and mitotic proliferation. A key downstream pathway involves GDNF-induced activation of the mTOR pathway, which is mediated by the MERK/ERK kinase pathway and requires differential phosphorylation of the mTORC1 regulator protein raptor at several sites including Serine863.
liferation. We also observed significant up-regulation of phosphorylation of the adaptor protein SHC1 at Ser^{222} in response to GDNF treatment. Binding of SHC1 to tyrosine^{1062} of the RET receptor is crucial for GDNF signaling (63), and Ser^{222} phosphorylation is the most common post-translational modification associated with SHC1 activation (56). Our data therefore indicate that phosphorylation of SHC1 at serine residues may participate in the regulation of SPC proliferation.

A previous study demonstrated that MAP2K1, the direct upstream kinase of ERK1/2 and MEK1 signaling, drives both GDNF- and bFGF-driven SPC self-renewal and proliferation (13). The same group also identified the Ras oncogene protein as an upstream phosphatase regulator of MEK1, based on the observation that germ cells that overexpress Ras proliferate in vitro without GDNF supplementation (18). Recent in vivo data have validated that MEK/ERK1/2 signaling in SPCs is independent of RA signaling and predominantly activated by GDNF, and contributes to cell cycle activity. Our phosphoproteome data demonstrate that sustained stimulation with GDNF results in sustained activation of the ERK1/2 kinase pathway, and its inhibition resulted in cell cycle changes and G2/M phase arrest. These data confirm that ERK1/2 signaling is a central pathway regulating SPC proliferation.

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is a key mediator of the cellular response to growth factors and regulates diverse processes including translation, ribosome biogenesis, mitochondrial metabolism, and autophagy (47, 64), and associated with longevity and translation, ribosome biogenesis, mitochondrial metabolism, rapamycin (mTOR) is a key mediator of the cellular response to growth factors and regulates diverse processes including translation, ribosome biogenesis, mitochondrial metabolism, and autophagy (47, 64), and associated with longevity and mitochondrial oxidative function (65). Our data indicate that mTOR signaling is strongly activated in SPCs in response to GDNF, with mTOR signaling being the enriched pathway in our data set. Activity of the mTOR complex 1 (mTORC1) is both positively and negatively regulated by interaction with its downstream effector raptor(48), in turns, mTORC1 activity depends on the phosphorylation of raptor (66). Our data show that in SPC, at least 4 residues of raptor can become phosphorylated, and we observed a significant up-regulation of Ser^{863}, Ser^{869} and Thr^{853} phosphorylation following GDNF refreshment. Raptor Ser^{863} phosphorylation functions as a master biochemical switch that modulates hierarchical raptor phosphorylation (50), and is required for nearby amino acid site phosphorylation at Ser^{859} and Ser^{855}. Significantly in the present study, the growth of cells was distinctly stepped up when we applied strategies to over expressed total raptor or sustained phosphorylation of raptor in SPCs. Furthermore, according to phosphorylating motifs and corresponding kinases prediction, we found that ERK1/2, which acts as a downstream effector of RAS/MAPK, is the predominant kinase involved in raptor Ser^{863} phosphorylation. Although Raptor itself is a protein with ubiquitous expression, it might not be expected to be essential for the viability of all cells. It was reported that when specifically disrupted hepatic mTORC1 signaling by expressing Cre under the control of the liver-specific albumin promoter carrying a conditional allele of Raptor (Alb-Cre RaptorloxP/loxP mice), mice had normal liver, normal glucose tolerance and remained responsive to rapamycin treatment (65). In our present study, however, it indicates that a progressive loss of spermatogonial progenitor cells in the absence of raptor.

Multiple adult tissues depend upon appropriate mTORC1 signaling to control stem cell maintenance, proliferation, and tumor suppression (67). Our data thus expand the known role of mTOR in SPCs, in which this complex is a central determinant of the balance between self-renewal and differentiation. mTORC1 over-activation leads to SPC differentiation (68) and counterbalancing mechanisms in SPCs that suppress mTORC1 activity sufficiently to allow for self-renewal include transcriptional repression through ZBTB16 (69) or P53 (70), and post-transcriptional sequestration of mTOR in NANOS2-containing ribonucleoprotein complexes (71). Thus, multiple mechanisms are in place to ensure SPC homeostasis but also initial differentiation into spermatogonial progenitor cells, which represents an essential mitotic proliferation step to increase the number of spermatogonia.

In summary, our study provides, for the first time, a global analysis of phosphorylation events in SPCs in response to GDNF. This dataset provides the basis for further analyses of signaling networks regulating fate decision of male germline stem cells. Among these previously undescribed signaling networks, we have identified activation of mTORC1 signaling through ERK kinase-mediated phosphorylation of multiple sites of raptor protein as an important pathway for SPC proliferation.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE (30) partner repository with the dataset identifier PXD005756. The details: Project name: Quantification of the GDNF-regulated proteome and phosphoproteome in spermatogonial progenitor cells (SPCs); Project accession: PXD005756; Reviewer account: Username: reviewer26292@ebi.ac.uk; Password: KsThpG90. Annotated spectra for the whole proteome data and the phosphoproteome data can be viewed using the free MS viewer (72) (http://prospector2.ucsf.edu) with the search key pvgzwhlm7w and pu0bedfmib.

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