Germ Cell Nuclear Factor Is a Repressor of CRIPTO-1 and CRIPTO-3*

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The pluripotency of embryonic stem and embryonic carcinoma cells is maintained by the expression of a set of “stemness” genes. Whereas these genes are down-regulated upon induction of differentiation, the germ cell nuclear factor (GCNF) is transiently up-regulated and represses several pluripotency genes. CRIPTO-1, a co-receptor for the morphogen nodal, is strongly expressed in undifferentiated and is rapidly down-regulated during retinoic acid-induced differentiation. Although CRIPTO-1 is expressed very low levels in adult tissues under normal conditions, it is found highly expressed in a broad range of tumors, where it acts as a potent oncogene. We show that expression of CRIPTO-1 is directly repressed by GCNF during differentiation. CRIPTO-1 is bound to a DR0 element of the CRIPTO-1 promoter in vitro, as shown by electrophoretic mobility shift assays, and in vivo, as demonstrated by chromatin immunoprecipitation. Reporter gene assays demonstrated that GCNF-mediated repression of the CRIPTO-1 promoter is dependent upon the DR0 site. Overexpression of GCNF in NT2 cells resulted in repression of CRIPTO-1 transcription, whereas expression of the transcription-activating fusion construct GCNF-VP16 led to an induction of the CRIPTO-1 gene and prevented its retinoic acid-induced down-regulation. Furthermore, we demonstrated that CRIPTO-3, a processed pseudogene of CRIPTO-1 on the X chromosome, is expressed in undifferentiated NT2 cells and is regulated by GCNF in parallel to CRIPTO-1. Thus, our study supports the hypothesis of GCNF playing a central role during differentiation of stem cells by repression of stem cell-specific genes.

Germ cell nuclear factor (GCNF), NR6A1, RTR is a member of the nuclear receptor superfamily of transcription factors (1) and acts as a transcriptional repressor (2, 3). Its interaction with several co-repressors has been demonstrated (4, 5). GCNF binds as a homodimer (6, 7) to DNA sequences termed DR0 elements, which are repeats of the sequence AGGTCA without spacing (8, 9).

GCNF is expressed in ectodermal structures and the primitive streak of the embryo at embryonic day (E) 6.5 (10). At E9.5 expression becomes more restricted to the developing nervous system and is drastically down-regulated at day E10.5. In the adult animal, GCNF is mainly expressed in germ cells (8). Targeted disruption of GCNF in mice resulted in embryonic lethality around E10.5 (11). Likewise, knockdown of GCNF in Xenopus embryos caused severe developmental defects (12). Conditional ablation of GCNF in the ovaries revealed an important function for fertility in adult animals (13).

GCNF is also expressed in embryonic stem (ES) cells and in teratocarcinoma cell lines such as NT2 cells and P19 cells. It shows a peculiar expression pattern during retinoic acid (RA)-induced differentiation with an initial up-regulation followed by a gradual shutdown (14, 15). Recently, several genes that are repressed by GCNF during this process could be identified (5, 13, 16–19). The repression of pluripotency genes like NANOG and OCT4 by GCNF suggests that GCNF may have a central role in initiating differentiation of stem cells.

CRIPITO-1 was initially cloned from NT2 cells as a RA-downregulated gene (20). It is a member of the EGF-CFC gene family and was later known to be a co-receptor for nodal (21–24).

Although CRIPTO-1 is widely expressed during early developmental stages in a complex expression pattern, it becomes restricted to the heart, forming structures around E8.0 (25). It is indispensable for heart development and the specification of the anterior-posterior and left-right axis (25, 26). CRIPTO-1 is highly expressed in ES cells (27), where it is involved in cardiomyocytic differentiation and acts as a negative regulator of neurogenesis (28–30).

In addition to its expression during early development and in stem cells, CRIPTO-1 is a potent oncogene, which is reactivated in a broad range of tumors (for review see Ref. 24). Several pseudogenes of CRIPTO-1 reside in the human genome (31). One of these, CRIPTO-3, is located on the X chromosome and retains an open reading frame as well as a portion of the promoter region of CRIPTO-1 (32).

In this study, we demonstrate that GCNF binds directly to the CRIPTO-1 promoter and thus represses its transcription during RA-induced differentiation of NT2 cells. We further show that CRIPTO-3 is expressed and regulated in a similar

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§ The abbreviations used are: GCNF, germ cell nuclear factor; E, embryonic day; NT2, Ntera2/D1; RA, retinoic acid; ES cells, embryonic stem cells; HA, hemagglutinin; EMSA, electrophoretic mobility shift assay; for, forward; rev, reverse; Chip, chromatin immunoprecipitation; TRIF, transiently retinoic-induced factor.
manner. Our work strengthens the concept of GCNF as a regulator of stem cell differentiation by repressing genes specifically expressed in undifferentiated cells.

EXPERIMENTAL PROCEDURES

Plasmids—The pSPUTK plasmid used for in vitro translation of HA-tagged GCNF has been described previously (15). For expression of GCNF in mammalian cells, the cDNA of HA-GCNF was cloned into the pCMX vector (33) via HindIII/BamHI. The GCNF-VP16 cDNA was a kind gift from Dr. Austin Cooney and was cloned into the pCMX vector (33) via HindIII/BamHI. The expression of GCNF in mammalian cells, the cDNA of HA-GCNF has been described previously (15). For GCNF serum (14, 15) or 1/1000 dilution of anti-HA antibody (Chemicon) stored at 4 °C, double-stranded oligonucleotides were labeled using Klenow polymerase (New England Biolabs) with [α-32P]dATP (American Biosciences). Unincorporated nucleotides were removed by gel filtration on Sephadex G25 spin columns (Roche Applied Science). Unincorporated nucleotides were removed by gel filtration on Sephadex G25 spin columns (Roche Applied Science). Site-directed mutagenesis was carried out by PCR using the mut-Cripto oligonucleotides described below.

Electrophoretic Mobility Shift Assay (EMSA)—Single-stranded oligonucleotides (purchased from MWG Biotech) were annealed in 10 mM Tris-HCl, pH 7.5, 60 mM NaCl and stored at −20 °C. Double-stranded oligonucleotides had 5′ overhangs of four nucleotides on both strands. For EMSAs, double-stranded oligonucleotides were labeled using Klenow polymerase (New England Biolabs) with [α-32P]dATP (American Biosciences). Unincorporated nucleotides were removed by gel filtration on Sephadex G25 spin columns (Roche Applied Science). Labeled oligonucleotides were stored at 4 °C in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 60 mM NaCl. Binding reactions were performed in a total volume of 12 μl consisting of 20 mM HEPES, pH 7.4, 80 mM NaCl, 20 mM KCl, 2 mM dithiothreitol, 1 μg of Cot-1 DNA (Roche Applied Science), 1× Complete protease inhibitor mixture (Roche Applied Science), and 1 μl of reticulocyte lysate or cellular extract, if not stated otherwise. A SIS primer was added for an additional 30 min. Complexes were resolved by gel filtration on Sephadex G25 spin columns (Roche Applied Science). Unincorporated nucleotides were removed by gel filtration on Sephadex G25 spin columns (Roche Applied Science). Site-directed mutagenesis was carried out by PCR using the mut-Cripto oligonucleotides described below.

Cell Culture and Transfection—HEK293 cells were grown on 35-mm tissue culture dishes at 37 °C, 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum (Invitrogen) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells grown to ~80% confluence were co-transfected with the indicated amounts of pCMX-HA-GCNF and 1 μg of the luciferase reporter plasmids using the FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s instructions. Empty expression vector was used to keep the transfected amounts of DNA constant. Cells were harvested 24–36 h later. Ntera2/D1 (NT2) cells were grown at 37 °C with 5% CO2 in OptiMEM (Invitrogen) containing 10% fetal calf serum (Invitrogen) with 5% fetal calf serum (Invitrogen) containing 100 IU/ml penicillin and 100 μg/ml streptomycin. Differentiation of NT2 cells was induced by adding all-trans-retinoic acid (Sigma) to a final concentration of 1 μM. Transfection of NT2 cells was done using the Lipofectamine 2000 reagent (Invitrogen), following the instructions of the supplier, with the recommended amounts of DNA. To minimize cellular toxicity, cells were transfected when they were about 90% confluent, and the supernatant was stored at −80 °C. In vitro translation was performed using the SP6-polymerase TNT reticulocyte lysate system (Promega) according to the manufacturer’s instructions and stored at −70 °C.

Luciferase Assay—The luciferase activity of transfected cells was determined using the high sensitivity luciferase reporter gene assay (Roche Applied Science). Transfected cells were washed twice with phosphate-buffered saline, dissolved in 170
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**FIGURE 1. GCNF binds to a DR0 element of the CRIPTO-1 promoter.** A, in vitro translated HA-tagged GCNF binds to different DR0 elements. The DR0 element from the platelet-derived growth factor-

-\( ^{\text{wt}} \)-HA antibody. GCNF did not bind to a mutated probe (mut-CRIPTO: AGCTCAcccTCA). B, binding of GCNF to the CRIPTO probe was competed by adding unlabeled probe in the indicated excess to the labeled CRIPTO element. Addition of reticulocyte lysate (RL) served as a negative control. wt, wild type.

\( \mu l \) of the lysis buffer supplied with the kit, and transferred into 1.5-ml reaction tubes. Cell debris was pelleted at 17,000 \( \times g \). 50 \( \mu l \) of the supernatant was transferred into individual wells of a 96-well microtiter plate. Light emission was measured for 15 s in a luminometer (Microlumat LB96 P, EG&G Berthold). After the injection of 100 \( \mu l \) of luciferase reagent, light emission was integrated over time. Each experiment was performed in triplicates and repeated at least three times.

**Northern Blot—** Total RNA was isolated from NT2 cells at the indicated time points with or without RA treatment using the RNAeasy kit (Qiagen) according to the manufacturer’s instructions. 5 \( \mu g \) of total RNA was separated by electrophoresis, blotted, hybridized, and washed following standard protocols. The Northern blot filters were hybridized with random primed [\( ^{32}P \)]dCTP-labeled cDNA probes (Megaprime labeling kit, Amersham Biosciences) corresponding to full-length human CRIPTO-1 (GenBank\( ^{\text{TM}} \) accession number gi: 45709269) and a commercially available human \( \beta \)-actin probe (Clontech). After hybridization and exposure, filters were stripped in distilled boiling water containing 0.1% SDS and rehybridized after controlling the stripping efficiency.

**Chromatin Immunoprecipitation (ChIP)—** 1 \( \times 10^{7} \) NT2 cells were induced with 1 \( \mu M \) RA for 20 h. Then cross-linking was accomplished by the addition of 37% formaldehyde to the medium to a final concentration of 1%. After 10 min at 37 \( ^{\circ} \)C cells were washed with ice-cold phosphate-buffered saline and transferred into 1.5-ml tubes. After brief centrifugation, the pellet was resuspended in lysis buffer (50 mm Tris-HCl, pH 8.1, 10 mm EDTA, 1% SDS) containing Complete protease inhibitors (Roche Applied Science). After rotation for 20 min at 4 \( ^{\circ} \)C, the lysate was sonicated on ice (UP50H, Dr. Hielserch GmbH) with a micro-tip probe (36 cycles, 30% max, 50% cycle) and pelleted (10 min, 13,000 \( \times g \), 4 \( ^{\circ} \)C). The supernatant (500 \( \mu l \)) was collected. One hundred \( \mu l \) was kept as an input control. Two 200-\( \mu l \) aliquots were diluted with 1800 \( \mu l \) of dilution buffer each (17 mm Tris-HCl, pH 8.1, 150 mm NaCl, 1.2 ml EDTA, 0.01% SDS, 1% Triton X-100). For precipitation 30 \( \mu l \) of rabbit polyclonal GCNF antiserum was added. The preimmune serum served as a negative control. Incubation was performed on a rotating wheel overnight at 4 \( ^{\circ} \)C. Twenty \( \mu l \) of protein A-Sepharose (Amersham Biosciences) and 10 \( \mu l \) of Cot-1 DNA (Roche Applied Science) were added and incubated for at least 2 h at 4 \( ^{\circ} \)C. Protein complexes were washed six times with salt buffer (20 mm Tris-HCl, pH 8.1, 150 mm NaCl, 2 mm EDTA 0.1% SDS, 1% Triton X-100). Elution was performed twice by adding 150 \( \mu l \) of elution buffer (0.1 m NaHCO\( _{3} \), 1% SDS) for 15 min at 20 \( ^{\circ} \)C. Twelve \( \mu l \) of 5 mm NaCl were added to the eluate, and protein-DNA complexes were de-cross-linked at 65 \( ^{\circ} \)C.

**RESULTS**

GCNF binds to the CRIPTO-1 promoter in vitro—GCNF directly represses the genes OCT4 and NANOG, which play a role in keeping stem cells in an undifferentiated state (5, 19). To explore, whether GCNF has a more general role for cell differentiation, we screened promoter sequences of genes down-regulated during RA-induced NT2 cell differentiation for GCNF binding sites. We identified a nearly perfect DR0 motif directly upstream, at positions +219 to −207 relative to the initiation codon ATG of the human CRIPTO-1 gene (Ensembl transcript ID: ENST00000296145) (Fig. 4C). First, we tested whether the DR0 motif is a binding site for recombinant as well as endogenous GCNF in vitro in EMOSAs. In vitro translated HA-GCNF bound to the CRIPTO-1 probe as efficiently as probes containing motifs from established binding sites for GCNF, namely from OCT4 and a motif from the platelet-derived growth factor-\( \beta \) (PDGF-\( \beta \)) promoter (Fig. 1A). The presence of HA-tagged GCNF...
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GCNF in protein-DNA complexes was confirmed by a supershift of the complex either with a polyclonal anti-GCNF serum or with a monoclonal anti-HA antibody. In contrast, an oligonucleotide containing the DRO motif from the CRIPTO-1 promoter, in which the central AGG had been replaced by CCC, was not bound by GCNF. The specificity of the GCNF/CRIPTO-1 promoter interaction was further assessed by competition of the labeled probe with an excess of unlabeled elements. A 10-fold excess bound by GCNF. The specificity of the GCNF/CRIPTO-1 promoter interaction was further assessed by competition of the labeled probe with an excess of unlabeled elements. A 10-fold excess

FIGURE 2. TRIF containing GCNF binds the DRO element in the CRIPTO-1 promoter. A, endogenous TRIF complex from cellular extracts of NT2 cells bind to the CRIPTO probe but not to the mutated probe (mut). wt, wild type. B, addition of a polyclonal α-GCNF serum supershifted the TRIF complex bound to the CRIPTO probe, confirming that it contains GCNF. The formation of the TRIF complex on the OCT4 promoter probe served as a positive control. n.s., nonspecific.

polyclonal anti-GCNF antibody, the genomic region surrounding the DRO motif in the CRIPTO-1 promoter and in the OCT4 promoter, but not the ARSB locus, could be amplified by PCR (Fig. 4A). Precipitation with preimmune serum did not result in an enrichment of the CRIPTO-1 or the OCT4 promoter. Thus, we have not only confirmed binding of GCNF to the human OCT4 promoter in vivo but also established CRIPTO-1 as a GCNF target.

In Vivo Binding of GCNF to the CRIPTO-3 Promoter—Sequence analysis of the PCR product from the ChIP precipitate revealed a double peak G/A at position −168 relative to the start ATG in the 5′ untranslated region of CRIPTO-1 (Fig. 4B). The autosomal gene CRIPTO-1 has a highly homologous sequence on the X chromosome termed CRIPTO-3, which is currently believed to be a processed pseudogene (31). Interestingly, in addition to the CRIPTO-3 open reading frame, 0.7 kb of the upstream region is highly homologous to the CRIPTO-1 promoter. Compared with CRIPTO-1, only five nucleotides are exchanged, and a poly(T) stretch is shortened by two thy midines. Additionally, three nucleotide changes are found between the 5′-untranslated regions of CRIPTO-1 and CRIPTO-3. The predicted transcript of CRIPTO-3 with a continuous open reading frame has seven nucleotide changes, four of them coding, in comparison with CRIPTO-1. As the DRO binding site and the surrounding sequences are 100% conserved in the CRIPTO-3 upstream region, we reasoned that

FIGURE 3. DRO-dependent repression of the CRIPTO-1 promoter. A, GCNF represses the wild-type CRIPTO-1 promoter in a dose-dependent manner. B, the CRIPTO-1 promoter with a mutated DRO element is not repressed by GCNF. Cells co-transfected with a CRIPTO-1 promoter-luciferase reporter construct and the indicated amounts of GCNF were harvested 24 h later, and the luciferase activities were quantified (mean ± S.D.).
GCNF might also bind to the CRIPTO-3 promoter. Comparison of the sequence of the ChIP precipitate with the sequences of CRIPTO-1 and CRIPTO-3 revealed that the guanidine at position −168 corresponds to CRIPTO-1, whereas the adenine corresponds to the CRIPTO-3 sequence (Fig. 4C). As amplicons from both genomic templates were detectable in the ChIP precipitate, GCNF obviously binds to the DR0 element not only in the CRIPTO-1 but also in the CRIPTO-3 promoter in vivo.

RA-induced Recruitment of GCNF to the Promoters of CRIPTO-1 and CRIPTO-3—Next, we analyzed the binding of GCNF to the CRIPTO-1 and CRIPTO-3 promoters separately using gene-specific primers in response to RA treatment. Both the CRIPTO-1 and the CRIPTO-3 promoter could be amplified from the precipitate, confirming that GCNF binds to both promoters (Fig. 5). Furthermore, RA treatment of NT2 cells resulted in a strong induction of GCNF binding compared with untreated cells, indicating that RA promotes GCNF recruitment to the CRIPTO promoters.

Expression Analysis of CRIPTO-3—To analyze whether CRIPTO-3 is expressed in NT2 cells, CRIPTO-1- and CRIPTO-3-specific primers were designed. To eliminate the possibility of genomic contamination the RNA was treated with DNase I prior to reverse transcription. Indeed, both CRIPTO-1 and CRIPTO-3 could be amplified from a cDNA of uninduced NT2 cells (Fig. 6A) but not from RNA samples incubated without reverse transcriptase. The identity of the PCR products as CRIPTO-1 and CRIPTO-3 was verified by sequencing (data not shown). Like CRIPTO-1, CRIPTO-3 is rapidly down-regulated during differentiation in response to RA in NT2 cells (Fig. 6B). These data suggest that the highly homologous 0.7-kb upstream region is sufficient for parallel regulation of the two CRIPTO genes. Yet, quantification of the relative amounts of CRIPTO-1 and CRIPTO-3 transcripts in total NT2 cell cDNA by quantitative real-time PCR, using a dilution series of the specific templates, revealed that CRIPTO-1 transcripts are ~10 times more abundant than CRIPTO-3 transcripts (data not shown).

To analyze the expression profile of CRIPTO-1 and CRIPTO-3, a PCR was performed on cDNAs from different human tissues. The cDNAs from uninduced and induced NT2 cells served as a positive and negative control, respectively.
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**FIGURE 6.** CRIPTO-1 and CRIPTO-3 are expressed in undifferentiated NT2 cells. A, CRIPTO-1 and CRIPTO-3 could be amplified using gene-specific primers from cDNA prepared from DNase I-treated RNA but not from RNA samples. CRIPTO-1 and CRIPTO-3 could be amplified from cDNA prepared from DNase I-treated RNA when reverse transcriptase (RT) was added to the reaction but could not be amplified when reverse transcriptase was not added. B, as determined by quantitative real-time reverse transcription PCR, CRIPTO-1 and CRIPTO-3 (CR-1, CR-3) are rapidly down-regulated to nearly undetectable levels after 4 days upon treatment of NT2 cells with 1 μM RA. C, PCR primer pairs specific for either CRIPTO-1 or CRIPTO-3 were used to amplify cDNAs from uninduced NT2 cells, from RA-induced NT2 cells, and from a commercially available multiple tissue cDNA panel. Robust amounts of the CRIPTO-1 and CRIPTO-3 cDNA could only be amplified from uninduced NT2 cells. Trace amounts of CRIPTO-1 could be amplified from spleen and ovaries.

**FIGURE 7.** CRIPTO-1 and CRIPTO-3 are down-regulated in NT2 cells transfected with wtGCNF and up-regulated in cells transfected with GCNF-VP16. A, Northern blot hybridized with a CRIPTO-1 probe. Each lane contains total RNA (5 μg) from uninduced (lanes 1, 3, and 5) and RA-induced (lanes 2, 4, and 6) NT2 cell transfected with the empty expression vector pCMX or the indicated cDNAs and harvested after 48 h. B, Real-time PCR analysis using CRIPTO-1- and CRIPTO-3-specific primers (mean ± S.D.).

CRIPTO-1 and CRIPTO-3 could only be amplified from uninduced NT2 cells but not from induced NT2 cells. For CRIPTO-1 only very faint signals were obtained from the spleen and ovaries, whereas CRIPTO-3 could not be amplified from adult tissues (Fig. 6C). Thus, both CRIPTO-1 and CRIPTO-3 are predominantly expressed during early development and in undifferentiated cells.

**Overexpression of GCNF and GCNF-VP16 in NT2 Cells—** Overexpression of wild-type GCNF should result in down-regulation of CRIPTO-1 and CRIPTO-3 in NT2 cells. Likewise, expression of the fusion protein GCNF-VP16 (3), which activates instead of repressing GCNF-regulated genes, should lead to an up-regulation of CRIPTO-1 and CRIPTO-3 transcription. Therefore, we transfected both cDNAs separately into NT2 cells and harvested the cells after 48 h either with or without RA induction. Expression levels of CRIPTO transcripts were first checked by Northern blot, which revealed a substantial repression of CRIPTO transcription in the wtGCNF-transfected cells (Fig. 7A). In contrast, transfection of GCNF-VP16 resulted in a moderate up-regulation of CRIPTO transcription, consistent with its function as a transcriptional activator (Fig. 7A). Although induction with RA resulted in a rapid down-regulation of CRIPTO in NT2 cells transfected with empty pCMX vector or wtGCNF, expression of GCNF-VP16 largely prevented RA-induced down-regulation of CRIPTO (Fig. 7A).
Next we analyzed CRIPTO-1 and CRIPTO-3 transcription by real-time PCR, as northern blotting is not able to distinguish between CRIPTO-1 and CRIPTO-3. In accordance with the Northern blot results, we observed a repression of CRIPTO-1 and CRIPTO-3 by about 3-fold after transfecting wtGCNF (Fig. 7B). Transfection of GCNF-VP16 induced expression of CRIPTO-1 and CRIPTO-3 by more than 2-fold in uninduced NT2 cells. As described above, RA induction resulted in a strong repression of the CRIPTO genes in the cells transfected with the empty vector pCMX or with wtGCNF. In contrast, repression of both CRIPTO-1 and -3 was efficiently attenuated by overexpression of GCNF-VP16.

DISCUSSION

Repression of genes conferring stem cell properties, e.g. OCT4 or NT2, is an early event in the differentiation of pluripotent cells (35). GCNF, a transcriptional repressor that is rapidly up-regulated after RA-induced differentiation of ES cells and embryonic carcinoma cells (14, 15, 19), was recently found to repress OCT4 and NANOG (5, 19). As GCNF might be a key regulator in the initiation of differentiation, we sought to identify further target genes of GCNF. In this work we identify CRIPTO-1 and CRIPTO-3 as new targets of GCNF-dependent repression both in vitro and in vivo.

CRIPTO-1 serves as a co-receptor for the morphogen nodal, which is essential for embryonic development (36). It is down-regulated upon RA-induced differentiation of human NT2 cells (20). Although RA induction of NT2 cells is accompanied by down-regulation of CRIPTO-1 and favors a neuronal differentiation (37), CRIPTO-1 is transiently up-regulated during cardiomyocytic differentiation of ES cells (29). As CRIPTO-1−/− ES cells cannot differentiate into cardiomyocytes and shift toward neuronal differentiation (28), CRIPTO-1 seems to be a key regulator of cell fate choice during early differentiation processes. Although recent observations suggest that GCNF up-regulates neuronal differentiation (38), its role during cardiomyocytic differentiation of stem cells has not been studied thus far.

Binding of GCNF to the CRIPTO-1 promoter repressed the transcription of the reporter. This repression was critically dependent on a DR0 element and was absent after targeted mutagenesis of this element. Remarkably, the mutated promoter construct displayed a diminished basal activity compared with the wild-type promoter, suggesting that the direct repeat is also bound by activating nuclear receptors. DR0 elements can also be bound by other nuclear receptors (39), as shown for SF-1 and LRH-1, which bind to a GCNF site of the OCT4 promoter (5, 40); upon differentiation SF-1 and LRH-1 are down-regulated and replaced by GCNF. A similar mechanism of dual regulation may also apply to CRIPTO, with GCNF not only repressing transcription by recruiting co-repressors to the promoter but also by competing with activating nuclear receptors for the same binding site. It remains to be investigated, whether SF-1 and/or LRH-1 activate the CRIPTO genes.

Although the importance of CRIPTO-1 during development has been shown by Cripto-1 knock-out embryos, which are severely affected in axis formation and cardiac development (25, 26), its role in undifferentiated stem cells is less clear. Recombinant CRIPTO-1 displayed mitogenic effects on NT2 cells and CID9 cells, suggesting that it might enhance cellular proliferation (41, 42). This is in line with its function as a potent oncogene (22, 24, 30, 42–45). Overexpression of CRIPTO-1 has been shown in numerous human cancers, including germ cell tumors. Recently, other targets of GCNF-mediated repression, OCT4 and NANOG, were also found to be overexpressed in germ cell cancers (46–49), and induction of transgenic expression of OCT4 resulted in massive tumor development in mice (50). Thus reactivation and de-repression of pluripotency genes in later life are likely to be involved in establishing a malignant cell phenotype. For future studies it will be interesting to investigate whether GCNF might be inactivated in a subset of cancers and may thus be a candidate tumor suppressor gene.

As for other genes that are highly expressed in stem cells (51, 52), several intronless, processed pseudogenes of CRIPTO-1 are dispersed throughout the genome (31, 32). Although CRIPTO-2, -4, and -5 showed several alterations that disrupt their presumed open reading frames, CRIPTO-3 accumulated only a few nucleotide exchanges compared with CRIPTO-1, four of them coding, and retains an open reading frame. Although it is unclear how the CRIPTO-3 gene emerged, CRIPTO-3 also possesses 0.7 kb of the upstream promoter region of CRIPTO-1 with only minor deviations from the CRIPTO-1 promoter, in addition to the sequence of the reinserted, reverse transcribed mRNA of CRIPTO-1. CRIPTO-3 was expressed in undifferentiated NT2 cells, albeit at lower levels than CRIPTO-1. In adult tissues we did not find substantial expression of CRIPTO-1 or CRIPTO-3. Lower expression of CRIPTO-3 levels might be explained either by the loss of more distantly located enhancers or by the lower transcription efficiency of the intronless CRIPTO-3 gene. Like CRIPTO-1, CRIPTO-3 is down-regulated upon RA treatment. As GCNF binds to both promoters, GCNF presumably represses not only CRIPTO-1 but also CRIPTO-3 during RA-induced differentiation of NT2 cells. The physiological function of CRIPTO-3 is still unclear. Because of its high sequence similarity it may be functionally redundant to CRIPTO-1. This may explain why CRIPTO-1 mutants are less severely affected than nodal mutants (36).

In conclusion, this study demonstrates binding of GCNF to the CRIPTO promoters and repression of its transcription. This confirms the role of GCNF as an important repressor of genes specifically expressed in stem cells upon induction of differentiation. In addition to the pluripotency genes identified as GCNF targets in previous studies, CRIPTO-1, which is involved in early cardiomyocytic differentiation of stem cells, was identified as a GCNF-regulated gene. This might point toward a function of GCNF not only in initiating cell differentiation but also in cell fate determination.

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