STRA8 Shuttles between Nucleus and Cytoplasm and Displays Transcriptional Activity*5

Marianna Tedesco, Gina La Sala, Federica Barbagallo, Massimo De Felici, and Donatella Farini

From the Department of Public Health and Cell Biology, University of Rome “Tor Vergata,” 00173 Rome, Italy

Strα8 (stimulated by retinoic acid 8) encodes a protein crucial for mammalian germ cells entering into premeiotic stages. Here, to elucidate the still unknown STRA8 molecular functions, we studied the cellular localization of the protein in several cell types, including premeiotic mouse germ cells and stem cell lines. We reported distinct STRA8 localization in germ and stem cell types and a heterogeneous protein distribution in the cytoplasm and nucleus of such cells suggesting that the protein can shuttle between these two compartments. Moreover, we identified specific protein motifs determining its nuclear import/export. Furthermore, we demonstrated that in transfected cell lines the nuclear import of STRA8 is an active process depending on an N-terminal basic nuclear localization signal. Moreover, its nuclear export is mainly mediated by the Exportin1 (XPO1) recognition of a nuclear export signal. Significantly, we also demonstrated that STRA8 associates with DNA and possesses transcriptional activity. These observations strongly suggest that STRA8 can exert important functions in the nucleus rather than in the cytoplasm as believed previously, likely depending on the cell type and regulated by its nuclear-cytoplasmic shuttling.

Germ cells play a unique role as the carriers of genetic information between generations. They are the only cells able to divide meiotically and to halve their genetic material generating haploid cells. In the mouse ovary, oocytes begin meiosis during fetal development around 13.5 days post-coitum (dpc)2 (1), whereas in the testis the onset of meiosis is delayed until after birth (2).

Recent findings indicate that despite the different timing for the meiotic entry, male and female germ cells might share an identical meiotic initiation pathway in which retinoic acid (RA) induces Strα8 (stimulated by retinoic acid 8) gene expression in premeiotic germ cells (3–6). The Strα8 gene encodes a predicted 393-amino acid protein and was originally identified in a gene screening to detect genes that are up-regulated in P19 embryonal carcinoma cells in response to RA (7). A subsequent study reported that Strα8 is expressed in embryonic stem and germ cells and male germ cells of embryonic and adult mice (8). By using in situ hybridization analysis, Menke et al. (9) demonstrated that Strα8 is expressed in embryonic ovaries in an anterior-to-posterior wave that spans ~4 days, from 12.5 to 16.5 dpc. In male gonads, Strα8 is expressed in premeiotic postnatal germ cells (5, 6) rather than in embryonic germ cells (8). Strα8−/− female and male mice are infertile due to severe gametogenesis impairment (11–13). In particular, in female embryos lacking Strα8, the initial mitotic development of germ cells is normal, but they fail to undergo premeiotic DNA replication and meiotic chromosome condensation. In male mutant mice, the premeiotic DNA replication is conserved (11, 13), and germ cells are able to partly condense chromosomes and initiate meiotic recombination. They fail, however, to regularly continue over the leptotene stage of prophase I (13). Although all these studies reinforce the importance of Strα8 in gametogenesis and perhaps in stem cell physiology, the molecular functions of this protein remain unknown.

Intracellular localization and its dynamics represent important information to identify protein functions. Apart from the study by Oulad-Abdelghani et al. (8), in which STRA8 was localized in the cytoplasmic fraction of P19 stem cells, no clear information is available on the intracellular localization of this protein and its dynamics, in particular in premeiotic and meiotic germ cells.

Movement of ions, metabolites, and other small molecules through the nuclear pore complex occurs via passive diffusion, but the translocation of cargoes larger than ~40 kDa generally requires specific transport receptors (14). These transport receptors are central to the nuclear import and export steps of recognizing signal-bearing cargoes, interacting with the nuclear pore complex, and delivering the cargo to its destination compartment. The largest group of transport receptors includes structurally related members of the karyopherin-β/importin-β (Kapβ/Impβ) protein family (importins, exportins, or transportins) that usually bind to specific signals within the cargo protein termed nuclear localization signals (NLS) or nuclear export signals (NES), respectively. These have classically been defined as primary amino acid motifs that are both necessary and sufficient for transport. Importin-α recognizes the NLS and forms a ternary complex with importin-β to enter into the nucleus, whereas exportins recognize the NES in the cargo protein, and the complex is exported from the nucleus by binding with the GTP-bound form of the guanine nucleotide-binding protein Ran
Nuclear-Cytoplasmic STRA8 Shuttling

(RanGTP) (15). The classical NES sequence, a short leucine-rich motif, is specifically bound by the exportin known as exportin 1 (XPO1 or CRM1) (16).

XPO1 binds export cargo proteins and RanGTP in the nucleus to form an export complex that is subsequently translocated to the cytoplasm where it dissociates by the RanGTP-Pase-activating protein action (16–18). Nuclear-cytoplasmic shuttling plays an important role in regulating the activity of several proteins involved in cell proliferation, transformation, and tumorigenesis, and signal transduction (19, 20). For example, numerous transcription factors are held inactive in the cytoplasm until adequate signals trigger their import to the nucleus and allow activation or repression of their respective target genes. Moreover, the nuclear export machinery also counteracts the slow but steady leakage of cytoplasmic proteins into the nuclear compartment.

It was hypothesized that in germ cells and stem cells the regulation of nuclear transport of transcription factors and machinery components could represent an important driver for differentiation (21). For example, importin proteins show a distinct localization pattern through ovary and testis development (22), and it has been shown that Importin13 (Ipo13), a member of importin-β gene family, plays a stage-specific role in nuclear-cytoplasmic translocation of cargoes that accompanies meiotic differentiation of the mouse germ cells (23).

STRA8 protein has a predicted 46-kDa mass and from the cytoplasmic compartment could passively enter the nucleus. Here, however, we show that this is not the case. In fact, we describe for the first time distinct cellular localizations of this protein in female and male premeiotic germ cells and in embryonic stem and embryonic carcinoma cell lines and identified specific protein motifs regulating its nuclear import/export.

The observation that STRA8, previously described as a prevalent in female and male premeiotic germ cells and in embryonic stem and embryonic carcinoma cell lines and identified specific protein motifs regulating its nuclear import/export. The STRA8 fragments were amplified by PCR using primers listed in Table 1 (primers 11–3, 11–14, 11–7, and 12–3), and subcloned into pBIND-COOH-STRA8 (aa 1–207), and pBIND-COOH-STRA8 (aa 209–393) vectors.

**EXPERIMENTAL PROCEDURES**

Localization of STRA8 in Primordial Germ Cells (PGCs), Spermatogonia, ESD3, and ECF9 cells—For immunocytochemical studies, PGCs were obtained from testes and ovaries of 13.5 dpc ovaries using primers 1–3 and 2–3 listed in Table 1. Plasmids expressing the fusion protein GFP-STRA8 and myc-STRA8 were constructed by subcloning in the coding sequence of mouse Str8 to the C terminus of pEGFP-C1 (Clontech) and pCDNA3-N2myc (Stratagene) using restriction enzymes EcoRI and Sall. GFP-HEK293 cells (ATCC), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. PGCs and spermatogonia were left to adhere to poly-L-lysine-coated gelatin-coated dishes. When indicated, leukemia inhibitory factor (Immunological Science, Naples, Italy) on 2-mercaptoethanol (Sigma), and 1000 units/ml leukemia inhibitory factor (Immunological Science, Naples, Italy). The plasmid encoding yellow fluorescent protein-XPO1 (YFP-XPO1) was generously provided by Dr. J. A. Rodríguez (University of Basque Country).

**Localization of Recombinant Wild-type (WT) STRA8 and Mutant STRA8 in GC-1 and HEK293 Cells—**GC-1 and HEK293 cells (ATCC), were grown in Dulbecco’s modified (ATRA) for 24 h. Mouse F9 embryonic carcinoma cells (ECF9, ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. PGCs and spermatogonia were left to adhere to poly-L-lysine-coated gelatin-coated dishes before fixation. All cell types were fixed by 4% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized for 10 min in 0.1% Triton X-100 in PBS. After a 1-h block in 5% bovine serum albumin in PBS, rabbit IgG or a polyclonal antibody against STRA8 (Abcam) was added at a 1:250 dilution in 0.5% bovine serum albumin in PBS and incubated overnight at 4 °C. A goat anti-rabbit secondary antibody (Alexa Fluor 488 or 568, Molecular Probes) was added to the samples for 1 h, and nuclei were labeled with Hoechst 33349 (1 μg/ml). Samples were visualized under a Leica CTR600 microscope.

**DNA Constructs—**The coding region of mouse Str8 (GenBank accession number NM 0092921) was amplified by reverse transcription-PCR from 1 μg of total RNA obtained from 13.5 dpc ovaries using primers 1–3 and 2–3 listed in Table 1. Plasmids expressing the fusion protein GFP-STRA8 and myc-STRA8 were constructed by subcloning the coding sequence of mouse Str8 to the C terminus of pEGFP-C1 (Clontech) and pCDNA3-N2myc (Stratagene) using restriction enzymes EcoRI and Sall. GFP-HEK293 cells (ATCC), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. PGCs and spermatogonia were left to adhere to poly-L-lysine-coated gelatin-coated dishes. When indicated, leukemia inhibitory factor (Immunological Science, Naples, Italy). The plasmid encoding yellow fluorescent protein-XPO1 (YFP-XPO1) was generously provided by Dr. J. A. Rodríguez (University of Basque Country).

**Localization of STRA8 in Primordial Germ Cells (PGCs), Spermatogonia, ESD3, and ECF9 cells—**For immunocytochemical studies, PGCs were obtained from testes and ovaries of 13.5 dpc CD-1 mouse embryos following MiniMACS immunomagnetic cell sorter method (PGCs purity >90%) (24). Spermatogonia were obtained from 7 days postpartum CD-1 mice, as reported previously (25). Mouse embryonic stem cells (ESD3, ATCC) were cultured in the presence of mouse embryonic fibroblasts in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% fetal calf serum (Invitrogen), 1 mM sodium pyruvate, 1× nonessential amino acids, 10–4 M 2-mercaptoethanol (Sigma), and 1000 units/ml leukemia inhibitory factor (Immunological Science, Naples, Italy) on gelatin-coated dishes. When indicated, leukemia inhibitory factor was substituted with 1 μM all-trans-retinoic acid

**TABLE 1 Primer used in this study**

| Oligonucleotide | Sequence 5’ to 3’ |
|-----------------|-----------------|
| 1) GFP-STRA8-up | AGGAATTCTATGGCCACCCCTGGAG |
| 2) myc-STRA8-up | AGGAATTCTGCGGACCACCCCTGGAG |
| 3) GFP-STRA8-dw | AGGGATCCATCCGGAATTCATG |
| 4) GFP-myc-HLH-dw | AGGAATTCTAAGCAGACCATGGACCTC |
| 5) GFP-ΔHLH-up | AGGTCGACCTCCTCTGGATTTTCTGA |
| 6) GFP-NE1-up | AGGAATTCTCTGTCACAGGCCCGCAT |
| 7) GFP-NE1-dw | AGGTCGACCTTATCCAGCTTTCTTCC |
| 8) GFP-NE2-up | AGGAATTCTGGTGAAGAGAGAGGTA |
| 9) GFP-NE2-dw | AGGTCGACTTACAGATCGTCAAAG |
| 10) GFP-NE3-up | AGGAATTCTCTCCTCTGAGATTTCCT |
| 11) pBINDSTRA8up | AGGAATTCTGCGGACCACCCCTGGAG |
| 12) pBIND-COOH | AGGAATTCGACCCCTGGATTTCTAG |

**Primers used in this study**

| Primer          | Oligonucleotide |
|-----------------|-----------------|
| 1) GFP-STRA8-up | AGGAATTCTATGGCCACCCCTGGAG |
| 2) myc-STRA8-up | AGGAATTCTGCGGACCACCCCTGGAG |
| 3) GFP-STRA8-dw | AGGGATCCATCCGGAATTCATG |
| 4) GFP-myc-HLH-dw | AGGAATTCTAAGCAGACCATGGACCTC |
| 5) GFP-ΔHLH-up | AGGTCGACCTCCTCTGGATTTTCTGA |
| 6) GFP-NE1-up | AGGAATTCTCTGTCACAGGCCCGCAT |
| 7) GFP-NE1-dw | AGGTCGACCTTATCCAGCTTTCTTCC |
| 8) GFP-NE2-up | AGGAATTCTGGTGAAGAGAGAGGTA |
| 9) GFP-NE2-dw | AGGTCGACTTACAGATCGTCAAAG |
| 10) GFP-NE3-up | AGGAATTCTCTCCTCTGAGATTTCCT |
| 11) pBINDSTRA8up | AGGAATTCTGCGGACCACCCCTGGAG |
| 12) pBIND-COOH | AGGAATTCGACCCCTGGATTTCTAG |
Eagle’s medium with 10% fetal calf serum. 2.5 × 10⁵ cells were transfected with 1.5 μg of pEGFP-STRA8 constructs (WT or mutants) or empty vector (pEGFP-C1) or pCDNA3-N2-myc-STRA8 constructs using TransFast transfection reagent (Promega) according to the manufacturer’s protocol. Twenty four hours after transfection, the cells were fixed with 4% (v/v) paraformaldehyde for 10 min. For myc-STRA8 detection, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Following a blocking step with 10% goat serum in PBS for 45 min, the c-Myc antibody (1:500; 9E10, Santa Cruz Biotechnology) was applied for 1 h at room temperature. After washing with PBS, cells were incubated with goat anti-mouse secondary antibody (Alexa Fluor 568, Molecular Probes) for 45 min. Nuclei were labeled as above. In the case of leptomycin B (LMB) treatment, at 24 h after transfection, the cells were treated with 6 ng/ml LMB for 3 h at 37 °C before fixation. Three samples each of about 100 fluorescent cells were counted and scored for subcellular localization in three independent experiments.

Identification and Mutation of Putative STRA8 NLS and NES —
The WoLF PSORT program (26) was used to identify sequences in murine STRA8 containing highly charged basic amino acid residues (Fig. 5A, indicated in boldface) that could potentially function as NLS. For generation of basic amino acid mutation (Arg to Ala) of pEGFP-STRA8 (Fig. 5B, GFP-STRA8-NSmut), a QuickChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer’s protocol with specific primers as follows: NLSR28A_R31A, 5′-tgtacagcttgcaagctggttgcgcaggacagttcaagtg-3′, and NLSR28A_R31A_antisense, 5′-cggggccgaggtcctgagtttg-3′; NES1F92A_L94A, 5′-gtgtaagcgttggcataccgttcaaattccatgaggt-3′; NES2L216A_F218A, 5′-gtgtaagcgttggcataccgttcaaattccatgaggt-3′; NES3:I356A_F358A, 5′-gtgtaagcgttggcataccgttcaaattccatgaggt-3′; and NES3:I356A_F358A_antisense, 5′-cttcttctttgctgaggtcctgcccaact-3′. NES motif prediction was achieved by using a Web-based NES motif predictor, NES Finder 0.2 (see Fig. 6B). A QuickChange site-directed mutagenesis kit was also used for generation of hydrophobic amino acids mutation to Ala of pEGFP-STRA8 (Fig. 5B, GFP-STRA8-NSmut, GFP-STRA8-NS2mut, GFP-STRA8-NS3mut, Fig. 7A) with specific primers as follows: NES1F92A_L94A, 5′-tgtcagctggcagctggttgcgcaggacagttcaagtg-3′, and NES1F92A_L94A_antisense, 5′-cggggccgaggtcctgagtttg-3′; NES2L216A_F218A, 5′-acaccttttttttcaaatggttagctgcacaacttc-3′, and NES2L216A_F218A_antisense, 5′-gcttcttttttttcaaatggttagctgcacaacttc-3′; NES3:I356A_F358A, 5′-gcttcttttttttcaaatggttagctgcacaacttc-3′, and NES3:I356A_F358A_antisense, 5′-gcttcttttttttcaaatggttagctgcacaacttc-3′. The DNA sequence of all mutants was confirmed at BMR Genomics.

Cell Fractionation and Western Blot Analysis —Total lysates were obtained from freshly isolated PGCs and spermatogonia. ESD3 and ECF9 cells were treated with ATRA (1 μM) for 24 h before lysis. GC-1 or HEK293 cells were lysed 24 h after transfection. Protein cellular extraction was performed in Lysis buffer (50 mM HEPES (pH 7.9), 15 mM MgCl₂, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mix (Sigma)). The homogenates were centrifuged for 10 min at 3,000 rpm to pellet nuclei. The supernatants were collected and used as cytosolic fractions. Nuclear fractions were extracted with buffer C (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 600 mM NaCl, 0.5 mM dithiothreitol, 10 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mix), followed by centrifugation for 15 min at 21,000 × g. Equal cellular amounts of each fraction were used for Western blot analysis. The indicated antibodies were diluted in TBST buffer (5% nonfat dry milk, 50 mM Tris-HCl, 150 mM NaCl (pH 7.5), 0.1% (v/v) Tween 20) and added to the polyvinylidene difluoride membranes for 1 h at room temperature or overnight at 4 °C followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 45 min at room temperature. All proteins were detected with ECL Plus detection reagents (Amersham Biosciences) and visualized by chemiluminescence.

Immunoprecipitation —GC-1 cells cultured 24 h after transfection with pCDNA3-N2 myc-STRA8 were washed with cold PBS and lysed (20 min at 4 °C) in buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 3% glycerol, 10 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mix (Sigma). Immunoprecipitation was carried with 1 μg of c-Myc antibody (9E10, Santa Cruz Biotechnology) or mouse IgG for 2 h at 4 °C. After washing, precipitates were analyzed by Western blotting using anti-XPO1 (BD Transduction Laboratories) or anti-c-Myc mouse monoclonal antibodies.

Cross-Linking Experiment —ECF9 and ESD3 cells were cultured in 60-μm dishes in the presence of 1 μM ATRA, and 5 × 10⁵ HEK293 cells were transiently transfected with 3 μg of pEGFP-STRA8. After 24 h, cells were treated or not for 10 min with 1% formaldehyde in the culture medium to cause molecular cross-linking according to a standard protocol. Cells were then collected by centrifugation and resuspended in 1 ml of TRIzol solution (Invitrogen). Following the protocol described in Ref. 27, the aqueous phase that contains RNAs was discarded, and DNA (with or without cross-linked proteins) and protein fractions were recovered in the interphase and phenol phase, respectively, after sequential precipitation following the manufacturer’s instruction. To isolate DNA-bound proteins, the DNA fraction was resuspended in TE (10 mM Tris, 1 mM EDTA) and treated with 30 μg/ml DNase for 30 min at 37 °C and sonicated. Total and DNA-bound protein fractions were then diluted in SDS-sample buffer for Western blot analysis with either anti-ERK42/44 (Santa Cruz Biotechnology) or anti-STR8 antibodies.

One-hybrid Transcription Activation Assay —4 × 10⁵ HEK293 cells were seeded in 24 wells and co-transfected with 200 ng of GAL4 reporter plasmid (pG5-luc, Fig. 9B), 200 ng of each pBIND fusion construct, and 200 ng of pCDNA-3 to make final 600 ng of DNA. For positive control experiments, cells were transfected with 200 ng of pACT-myoD containing the VP16 activation domain and 200 ng of pBIND-Id control vector of CheckMate™ mammalian two-hybrid sys-
tem (Promega) instead of pBIND vector. Each well also received 10 ng of a pRL-TK vector (Promega) to normalize for transfection efficiency. At 48 h after transfection, cells were washed three times with PBS and scraped in 100 μl of reporter lysis buffer (Promega). Luciferase activity in 20 μl of the cell extracts was quantified using the Dual-Luciferase reporter assay system (Promega). Protein concentration was determined using a BCA protein assay kit (Pierce). Each extract was assayed three times with a Fluoroskan Ascent FL luminometer. The Firefly luciferase activity was divided by the Renilla luciferase activity, and transcriptional activity was expressed as fold increase over the pBIND (GAL4-DBD alone) control group.

RESULTS

Intracellular Immunolocalization of STRA8—Cellular immunolocalization showed positivity for STRA8 in about 30% of PGCs freshly isolated from 13.5-dpc ovaries, whereas male PGCs of the same age were immunonegative. Somatic cells of both sexes were STRA8-negative (Fig. 1A). These observations are consistent with the notion that at this age female PGCs are entering into meiosis, whereas male PGCs are undergoing a
mitotic block in G₀/G₁ (2). Although in about 55% of the STRA8-positive PGCs, STRA8 was evenly localized in the cytoplasm and nucleus (Fig. 1, upper panel, and C), the protein appeared prevalently nuclear in the rest of the cells (Fig. 1, upper panel, and C). The specificity of the antibody was confirmed by immunoblotting (Fig. 1B).

In 7 days postpartum spermatogonia, distinctive STRA8 immunolocalization was observed (Fig. 1, lower panel, and C). About 70% of spermatogonia in the whole population were STRA8-positive, and of these 41% showed the protein immunopositivity exclusively in the cytoplasm, whereas in the remaining a diffuse distribution both in the cytoplasmic and the nuclear compartment was detected. No STRA8 immunopositivity in somatic cells was observed.

Because STRA8 protein was first identified as a retinoic acid-responsive protein in various stem cell types (8), we performed intracellular immunolocalization of the protein also in ESD3 and ECF9 cells cultured for 24 h in the absence or presence of 1 μM ATRA. As expected, STRA8 protein was immunodetectable only when cells were stimulated with ATRA (Fig. 2, A and B). In both ECF9 and ESD3 cells, STRA8 was exclusively nuclear, and prolonged ATRA incubation for 48 or 72 h did not change the protein localization (data not shown).

STRA8 Can Shuttle between Nucleus and Cytoplasm—To verify whether STRA8 can shuttle between nucleus and cytoplasm and to identify the localization signals, we produced a GFP-STRA8 fusion protein and transiently expressed the construct in different cell types. In GC-1 cells, the only available germ cell line (SV40 T-large antigen immortalized spermatogonia-like cells (28)), in which no STRA8 expression was detectable (data not shown), we observed that GFP-STRA8 localization was heterogeneous with a prevalent presence in the cytoplasm (45%) or throughout the cytoplasm and nucleus (38%) and with the 17% of the transfected cells showing the protein exclusively in the nucleus (Fig. 3, A and B). A similar heterogeneous GFP-STRA8 compartmentalization was observed in other transfected non-germ cell types (Fig. 3B). Immunoblotting of transfected GC-1 cell cytoplasmic and nuclear fraction lysates probed with anti-GFP antibody (Fig. 3C) and anti-STRA8 antibody (data not shown) confirmed the heterogeneous distribution of STRA8 in such cells.

Because the fusion with GFP may change the biological function of STRA8 because of larger molecular mass (70 kDa instead of 46 kDa) and the fusion with a tag could affect the localization of several proteins (29), we constructed N-terminal Myc-tagged STRA8 expression vector and transfected the construct into both GC-1 cells and HEK293 cells (cells showing very high transfection efficiency). Immunolocalization (supplemental Fig. S1) and immunoblotting analysis (data not shown) gave results comparable with those obtained with the GFP-STRA8 construct.

Nuclear Localization of STRA8 Is Mediated by HLH Domain—The N-terminal HLH domain is a well conserved region of the Stra8 sequence (NCBI GeneID 20899, aa 17–84; see also supplemental Fig. S2 and supplemental Fig. S3 in Ref. 12. This domain allows protein-protein interaction (30) and may include functional sequences for nucleus-cytoplasm shuttling of proteins (31), for example in Id2 (32) or in the steroid receptor co-activator 3 (SRC-3) (33). To investigate whether the HLH region of STRA8 plays a functional role in its localization, we generated an N-terminal mutant construct deleted in the HLH domain (aa 1–84) and fused to GFP (GFP-H9004HLH-STRA8, schematic representation in Fig. 4A). The correct expression of the mutant protein was verified by immunoblotting with anti-GFP antibody in the total cell lysates obtained from transfected GC-1 cells after 24 h of culture (supplemental Fig. S3). As shown in supplemental Fig. S3, we observed a remarkably higher steady-state level of this STRA8 mutant protein (∆HLH)
than of the WT STRA8 when equal amounts of plasmid DNA were transfected into the cells. Moreover, an additional higher molecular weight band in the ΔHLH mutant lysates, probably corresponding to a covalent structural modification of the STRA8 protein, was observed. Similar results were obtained in HEK293 and HeLa cell lines (data not shown). When GFP-ΔHLH-STRA8 was transfected in GC-1 cells, a prevalent cytoplasmic localization of the mutant protein in all transfected cells was observed (Fig. 4B). A similar result was obtained in HEK293 and HeLa cell lines (data not shown). This distinctive
localization of the ΔHLH mutant in comparison with the WT, confirmed by Western blot analysis using anti-GFP antibody (Fig. 4C), suggests that the HLH domain contains a signal that facilitates its nuclear localization or that is necessary to mediate an interaction of STRA8 with an unknown protein promoting its nuclear translocation.

**Putative Nuclear Localization Sequences in HLH Domain of STRA8**—In the aim to discriminate between the possibilities reported above, we used the WoLF PSORT program (26), which allows us to identify sequences in proteins containing highly charged, basic amino acid residues that could potentially function as NLS. Canonical NLS consist of two classes, including monopartite NLS, composed of a single stretch of basic amino acids (34), and bipartite NLS, consisting of two basic residues, a spacer of ~10 amino acids, and a second region consisting of at least three out of five basic residues (35). We identified a putative nuclear localization signal located at the N-terminal region (amino acid residues 28–33) and occurring within the HLH domain. This sequence is a monopartite cluster, and the basic amino acids are conserved among different STRA8 homologs (Fig. 5A). We first examined the subcellular localization of the GFP fusion protein containing only the HLH domain of STRA8, and we observed that the protein was uniformly distributed in both cytoplasm and nucleus (data not shown). To check if the failed accumulation into the nuclear compartment of the fusion protein was due to the proximity of GFP to the putative NLS sequence, we transfected GC-1 cells with myc-HLH-STRA8. The immunolocalization of the fusion protein with anti-Myc antibody showed that myc-HLH-STRA8 was exclusively present in the nucleus of the cells (supplemental Fig. S4) indicating that in the HLH domain, a functional NLS is present. To test whether the NLS in the HLH domain is also necessary to induce nuclear import of STRA8, we converted all the basic arginine residues in the sequence (Arg-28 and Arg-31 to Arg-33) to alanine (schematic representation in Fig. 5B) and analyzed the distribution of the GFP-STRA8-NLS mutant in transiently transfected GC-1 and HEK293 cells. The correct expression of the mutant protein was verified by immunoblotting with anti-GFP antibody in the total cell lysates obtained from transfected GC-1 cells after 24 h of culture (data not shown). As shown in Fig. 5B, bottom panel, the GFP-STRA8-NLS mutant localized exclusively in the cytoplasm, confirming the requirement for the RVVRR sequence motif for STRA8 nuclear import.

**STRA8 Is Actively Exported from Nucleus through XPO1 Recognition of an NES Sequence**—The exit of proteins from the nucleus is mainly regulated via the nuclear export receptors. The XPO1 protein, also known as exportin1 and CRM1, is the most versatile of all export receptors, being involved in the movement of many different classes of proteins (17). For this reason, we tested the effect of the specific XPO1-mediated nuclear export inhibitor LMB (36) on the cellular localization of transiently expressed GFP-STRA8 protein in GC-1 and HEK293 cells. Fluorescence imaging in GC-1 cells showed that as expected LMB treatment (6 ng/ml for 3 h) did not change the diffuse nuclear and cytoplasmic localization of the GFP protein (data not shown). However, LMB caused a marked redistribution of GFP-STRA8 with over 95% of the transfected cells showing an increase in its nuclear localization (Fig. 6A). The relocalization of GFP-STRA8 to the nucleus was already evident after

---

**FIGURE 5. NLS in the HLH domain mediates STRA8 nuclear localization.** A, NLS sequence (boxed) with the basic amino acids (boldface) is conserved in the STRA8 protein from different vertebrate species. The monopartite basic NLS consensus present in SV40-Large Antigen was shown (34). B, top, schematic representation of the GFP-STRA8-NLS mutant fusion protein. Basic amino acids (arginine) were muted in alanine. Bottom, subcellular localization of GFP-STRA8 or GFP-STRA8-NLSmut in GC-1 cells after 24 h of transfection as determined by fluorescence microscopy. The mutated NLS protein is clearly localized prevalently in the cytoplasm in comparison with WT STRA8.
FIGURE 6. Nuclear export of STRA8 is dependent on XPO1 and NES sequence. A, XPO1-mediated nuclear export inhibitor LMB restrains STRA8 localization in the nucleus. GC-1 cells transiently transfected with GFP- Stra8 plasmid and cultured for 24 h were further incubated without (−LMB) or with 6 ng/ml of LMB (+LMB) for 3 h. The presence of LMB induced a relocalization of GFP-STRA8 to the nucleus. B, identification of STRA8 NES. Three potential NES in Stra8 sequence was identified with a Web-based NES motif predictor, NES Finder 0.2. In the consensus NES sequence, Φ indicates a large hydrophobic residue, such as leucine, isoleucine, valine, or methionine. The RevNES that represents the well characterized NES model and the sequences of other knowing NES in different proteins are shown. C, comparison of putative NES in STRA8 homologs of various species. Residues critical to NES activity are indicated in boldface. D, left, schematic representation of the GFP-STRA8-NES fusion proteins used for GC-1 cells transfection. Right, localization of the corresponding constructs in transfected GC-1 cells treated (+LMB) or not (−LMB) with LMB for 3 h. Sequences between amino acids 174 and 348 (present in GFP-NES2 construct) were crucial for STRA8 cytoplasmic localization.
30 min of treatment and reached its maximum at 3 h. The kinetics of this LMB response is comparable with that observed for other nuclear shuttling proteins (37), supporting the possibility that STRA8 may be actively exported from the nucleus through an XPO1 pathway. LMB is a Streptomyces metabolite that inhibits export of leucine-rich NES-containing proteins preventing the association of XPO1 with such cargoes (36). Therefore, the strong effect of this drug on STRA8 localization suggests that this protein is exported from the nucleus through the XPO1/exportin recognition of an NES sequence. Examination of the mouse Stra8 sequence for a classical Rev-type NES consensus sequence (38) using NES software revealed the presence of three similar motifs that we refer to as NES1, NES2, and NES3 (Fig. 6B, upper panel) that display significant similarities to previously identified NES (Fig. 6B, lower panel) and include hydrophobic residues conserved in different species, suggesting evolutionary significance (Fig. 6C).

These sequences are included in the α-helical region of STRA8 protein (supplemental Fig. S2), a common structural characteristic of other well known NES motifs (39). To address the contribution of these sequences to the nuclear export of STRA8, we generated a number of GFP-stra8 proteins containing only one of the NES sequences as indicated in Fig. 6D (left panel). GC-1 cells were transfected with the expression plasmids and after 24 h were incubated with or without LMB (6 ng/ml) for 3 h. All these proteins were expressed at the expected size and at similar levels (data not shown). As shown in Fig. 6D (right panel), GFP-NES2 (aa 174–348) containing only the second NES sequence of STRA8 was distributed prevalently in the cytoplasm, and treatment with LMB caused a marked redistribution in the nucleus. The same result was obtained in HEK293 cells (data not shown). By contrast, GFP-NES1 and GFP-NES3 were uniformly distributed between the cytoplasm and nucleus in GC-1 cells, and they were not affected by LMB, indicating that these sequences do not contribute to STRA8 intracellular distribution. Thus, these experiments indicate that the region included between aa 174 and 348 containing the LMB-sensitive NES2 motif plays a major role in the STRA8 nuclear export.

The inability of the GFP-ΔHLH-stra8 and GFP-stra8-NLSmut containing the NES2 motif to localize within the nucleus of the transfected cells even in the presence of LMB (Fig. 6D, right upper panel, and data not shown) can be explained by the impossibility of entering into the nucleus and forming a complex with XPO1 rather than by the lack of nuclear retentive capacity. In fact, the GFP-NES2 protein that, because of its small dimension, is likely to enter the nucleus by diffusion is exported through an LMB-sensitive mechanism.
**STRA8 Interacts with XPO1 and Its Overexpression Increases STRA8 Nuclear Export**—The effects of LMB on STRA8 nuclear export and the nuclear accumulation of STRA8-NES2mut suggest that XPO1 might have a major role in the cytoplasmic localization of this protein. This aspect was further analyzed by overexpressing yellow fluorescent protein-tagged XPO1 in HEK293 cells co-transfected with myc-STRA8. After 24 h of culture, we observed that when expressed alone, myc-STRA8 was distributed both in the cytoplasm and nucleus of HEK293 (Fig. 8A, Untreated, left panel) and GC-1 cells (data not shown). By contrast, co-expression of YFP-XPO1 caused the exclusive cytoplasmic localization of myc-STRA8 (Fig. 8A, Untreated, right panel). Moreover, the overexpression of XPO1 in HEK293 cells could also revert the inhibitory effect of LMB on the nuclear localization of myc-STRA8 (Fig. 8A, LMB, right panel). Finally, to demonstrate physical interaction of STRA8 with XPO1, Myc-tagged full-length STRA8 was transfected into HEK293 cells to determine whether it would co-immunoprecipitate with XPO1 from cell lysates. Recombinant STRA8 expression was verified by SDS-PAGE and Western blotting to the input lysates with an anti-Myc antibody (Fig. 8B). The remaining lysates were subjected to immunoprecipitation with the same antibody followed by Western blotting with the XPO1 antibody. As shown in Fig. 8B, XPO1 co-immunoprecipitated with myc-STRA8 suggesting that the two proteins can interact and indicating that XPO1 allows regulated export of STRA8 from nucleus to cytoplasm.

**STRA8 Associates with DNA and Possesses High Transactivation Activity**—To test whether STRA8 can interact with DNA, we performed a protein-DNA cross-link assay. ECF9 and ESD3 cells, expressing endogenous STRA8 in the nucleus following ATRA stimulation and HEK293 cells transfected with GFP-STRA8, were cultured for 24 h, and DNA-protein complexes were fixed by adding 1% formaldehyde in the last 10 min of culture. Total and DNA-bound protein fractions isolated from the cells, as described in detail under “Experimental Procedures,” were then analyzed in Western blot for the presence of STRA8. As shown in Fig. 9A, both transiently expressed (upper panel) and endogenous STRA8 (lower panel) were recovered in the DNA-bound protein fractions and in the total protein fractions as well. When cells were not cross-linked, STRA8 was found exclusively in the total proteins fraction.

The nuclear localization of STRA8 and its possible association with DNA suggest that it may possess gene transcription activity. To verify such a possibility, STRA8 was fused to the yeast GAL4-DNA binding domain and transfected into HEK293 cells together with a GAL4 luciferase reporter plasmid containing five GAL4-DBD sites upstream of a TATA box (Fig. 9B, upper panel). As shown in Fig. 9B, WT STRA8 caused a highly significant concentration-dependent increase in GAL4 reporter gene transcription over the GAL4-DBD control. In contrast, STRA8 not fused to the GAL4-DBD had no effect on the luciferase transcription activation. This indicates that its binding to DNA is necessary for allowing transcription of the

---

**FIGURE 8.** A, XPO1 overexpression causes STRA8 to accumulate in the cytoplasm. HEK293 cells expressing myc-STRA8 alone or co-transfected with YFP-XPO1 were treated with or without LMB for 3 h, and after 24 h, immunofluorescence analysis was performed with anti-Myc antibody as described under “Experimental Procedures.” Left panel, in HEK293 cells, which overexpressed YFP-XPO1, myc-STRA8 is completely excluded from the nucleus. Right panel, when myc-STRA8 cells were co-expressed with YFP-XPO1, LMBs were not be able to induce its nuclear accumulation. B, STRA8 binds XPO1 in vivo. Protein from HEK293 cells expressing myc-STRA8 (lane 1) were immunoprecipitated (IP) with anti-Myc antibody (lane 3) or IgG (lane 2); STRA8 complexes were resolved, and immunoblots (IB) were probed for XPO1 and Myc.
reporter gene. To map the STRA8 domain responsible for the observed transcriptional activity, we fused different portions of STRA8 to the GAL4-DBD (Fig. 9C, left panel) and tested the ability of these chimeric proteins to activate the transcription of the GAL4-dependent reporter luciferase gene. We verified by Western blot analysis the expression of these fusion proteins (data not shown).

As shown in Fig. 9C (right panel), the GAL4-C-terminal region of STRA8 potently stimulated the activity of the GAL4-dependent reporter gene, and its effect is 2-fold the full-length STRA8 fusion protein. On the contrary, both the HLH and N-terminal region of STRA8 did not affect transcriptional activity.

**DISCUSSION**

Despite its essential role in germ cell development, it is not known how STRA8 controls the crucial decision of such cells to engage meiotic division. Similarly unknown is the role of STRA8 in retinoic acid-induced differentiation of stem cell lines. In general, a key characteristic of proteins, which can begin to elucidate their possible function, is their subcellular localization. In this study, we show for the first time that around 13.5 dpc when germ cells meiosis is beginning in the ovary, a subpopulation (about 30%) of female PGCs express STRA8, whereas male PGCs are negative for the protein. In about 55% of the STRA8-positive germ cells, the protein is present both in the nuclear and cytoplasmic compartments (Fig. 1, A and C), and in the remaining it accumulates prevalently in the nucleus. We speculate that the distribution of STRA8 in such cells may depend on distinct cellular conditions (for example, PGCs that have already entered the meiotic division or not) and reflect a different rate of movement through the nuclear envelope or a different retention activity by the two cellular compartments. Besides the nuclear-cytoplasmic localization, a restricted localization of STRA8 in the cytoplasm was observed in a relevant portion (about 40%) of the spermatogonial population obtained from 7 day-old testes (Fig. 1, A and C). At this age, spermatogonia consist of type A1–A4, intermediate and type B spermatogonia, and a minority of spermatogonial stem cells, whereas germ cells in the meiotic prophase are virtually absent (41). Because of such heterogeneity and the lack of specific markers for each cell type, we were unable to associate this distinctive STRA8 localization to a particular type of spermatogonia. Because, however, the same pattern of STRA8 distribu-
Nuclear-Cytoplasmic STRA8 Shuttling

The heterogeneous intracellular distribution of STRA8 in germ cells is indicative of its capacity to shuttle between the two compartments and probably of a different cell-dependent interaction with molecules that determine its final steady-state localization. Cell type-specific differences in terms of intracellular localization are common with a number of proteins, especially transcription factors, and in many cases are determined by a specific mechanism of regulation of cytoplasmic/nuclear shuttling. For example, specific phosphorylation is an efficient and potentially rapidly responsive mean of modulating NLS or NES accessibility (43).

To investigate the molecular mechanisms that determine the nuclear-cytoplasmic distribution of STRA8, we used epitope-tagged constructs of the protein and cell line transient transfection experiments. We demonstrated heterogeneous distribution of transfected GFP-STRA8 in the spermatagonia-like GC-1 cells and in other cell lines. Moreover, we identified NLS and NES sequences present in the STRA8 structure. The N-terminal region of STRA8 is predicted from sequence analysis to contain a well-conserved HLH domain (Fig. 5A and supplemental Fig. S2). Our data reveal that this region is necessary and sufficient for the nuclear import of the protein. In fact, an NLS sequence is present in this domain that when mutated or deleted impairs the STRA8 movement into the nucleus. We do not know, however, if the HLH domain, normally important for protein-protein interaction, mediates the direct binding of importins to NLS or other protein(s) that could indirectly lead nuclear STRA8 localization. The transfection experiments also showed that STRA8 is able to interact with XPO1 and that it accumulates in the nucleus when the XPO1-dependent export pathway is blocked with LMB. Moreover, its nuclear exclusion increases when XPO1-tagged protein is co-expressed in the cell (Fig. 8), thus indicating that the cytoplasmic presence of STRA8 is an active process and not a default localization of a cytoplasm-synthesized protein. The central region of the protein (aa 209–218) is important for the presence of a functional leucine-rich NES homolog to classical XPO1-bounded Rev-NES (38, 40). When fused to GFP, this region is able to locate the recombinant protein in the cytoplasm (Fig. 6D). In addition, disruption of this NES sequence by alanine substitution of hydrophobic leucine and phenylalanine residues (GFP-STRA8-NES2mut) abolishes the cytoplasmic localization of STRA8 in 95% of the transfected cells and induces its nuclear accumulation (Fig. 7). The other two putative NES motifs (aa 85–94 and 349–358) that we identified in the STRA8 sequence were nonfunctional. In fact, they were not able to cause the relocation of the protein when fused to GFP, and their mutation does not change the STRA8 intracellular distribution.

The active import of STRA8 into the nucleus raises the little considered possibility that this protein may act as transcription factor or cooperate with transcription factors in regulating specific gene activities. We actually obtained evidence strongly supporting such a possibility. In the stem cell lines in which endogenous STRA8 is prevalently nuclear and in transiently transfected cells showing heterogeneous GFP-STRA8 localization, we found that STRA8 associates with DNA when the cells are exposed to formaldehyde. Because this treatment cross-links both protein-DNA and protein-protein, it is possible that STRA8 can interact with DNA in an indirect manner. Such a possibility is supported by the observation that STRA8 does not possess a canonical basic DNA binding region next to its HLH domain (NCBI GeneID 20899, aa 17–84; see also supplemental Fig. S2 and supplemental Fig. 3 in Ref. 12). However, the presence of the basic NLS sequence in the first helical region of the HLH domain could mediate the direct DNA binding of STRA8. Finally, in the one-hybrid transcription activation assay, STRA8 fused to a DNA binding domain showed a surprisingly high capability to activate DNA transcription, and the C-terminal region seemed to be important for this action. In conclusion, we stress that the novel shuttling ability of STRA8 reported here may be a relevant mechanism underlying the regulation of its biological functions. Moreover, its ability to transactivate a reporter gene strongly suggests the intriguing possibility that STRA8 may act as a transcription factor or transcriptional co-regulator. The functional consequences of the shuttling activity of STRA8 and its specific target gene(s) remain to be established. STRA8 is indispensable for meiotic entry in embryonal female and postnatal male germ cells. Different STRA8 localization integrating different signals from the cytoplasmic and nuclear compartments could be important to coordinate nuclear and/or cytoplasmic events in the shift between mitosis and meiosis. STRA8 function seems, however, not restricted to meiosis because it is expressed also in stem cell lines following stimulation with RA, a well-known differentiating agent, and in testicular germ cell cancer. There is no information on the function(s) of STRA8 in these cell types in which meiotic events do not represent the normal differentiation pathway. An interesting possibility is that in such cells STRA8 might be involved in differentiation and cell cycle processes requiring spatial and temporal localization of the protein different from them involving STRA8 during the premeiotic stage. In this regard, it is interesting to note that the Strα8 promoter is expressed by neuronal cells (44) that represent a common RA-induced cell type in embryonic stem cells. Further studies are needed to clarify and dissect the molecular functions of STRA8 in premeiotic germ cells and in the RA-induced differentiating stem cells.

Acknowledgments—We are grateful to Dr. Rodriguez for the generous gift of YFP-XPO1; to Prof. C. Sette and Dr. A. Di Florio for fruitful discussions; to Dr. S. Di Siena and Dr. F. Barrios for the spermatogonial cell preparation; to Mr. G. Bonelli for the images preparation; and to Dr. I. Moscatelli for critical reading of the paper.

D. Farini, unpublished observations.
