Biochemical and Biophysical Research Communications 428 (2012) 422–426

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transcription-dependent nuclear localization of DAZAP1 requires an N-terminal signal

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ARTICLE INFO

Article history:
Received 12 October 2012
Available online 27 October 2012

Keywords:
DAZAP1
RNA-binding protein
Transcription-dependent nuclear localization
SLIRP

ABSTRACT

Deleted in Azoospermia Associated Protein 1 (DAZAP1) is a ubiquitous hnRNP protein required for normal development and spermatogenesis. It resides predominantly in the nucleus and moves between the nucleus and the cytoplasm via a ZNS shuttling signal at its C-terminus. DAZAP1 accumulates in the cytoplasm when RNA polymerase II activity is inhibited by actinomycin D. Here we report the mapping of a 42-amino acid segment (N42) at the N-terminus of DAZAP1 that is both necessary and sufficient for its transcription-dependent nuclear localization. In addition, using a yeast two-hybrid system, we have identified SLIRP as a N42-binding protein which may regulate DAZAP1 subcellular localization.

1. Introduction

DAZAP1, also known as proline-rich RNA-binding protein (PRRP), was originally isolated via its interaction with the Y chromosome-encoded spermatogenic factor Deleted in Azoospermia (DAZ) [1]. DAZAP1 is a ubiquitous RNA-binding protein and has been implicated in RNA splicing and translation [2–5]. During spermatogenesis, DAZAP1 is dynamically distributed in the testis where it is expressed most abundantly: it is first present at high levels in the nuclei of mid-pachytene spermatocytes, remains in the nuclei of round spermatids, and then relocates to the cytoplasm during spermatid elongation when nuclear transcription shuts down [6]. DAZAP1 is required for normal development and spermatogenesis, and its deficiency in mice results in perinatal death, growth retardation, and spermatogenic arrest [7]. DAZAP1 is a component of the hnRNP particle and shares several properties with hnRNP A1, an extensively studied hnRNP protein [8,9]. Both of these proteins contain two RNP-type RNA-binding domains (RBDs) in the N-terminal portion, though the C-terminal portions of DAZAP1 and hnRNP A1 are rich in proline and glycine, respectively [1]. DAZAP1 and hnRNP A1 are both predominantly present in the nuclei of somatic cells and shuttle between the nucleus and the cytoplasm. However, the ZNS shuttling signal at the C-terminus of DAZAP1 shares little sequence similarity with the M9 shuttling signal in hnRNP A1 [10]. In the presence of actinomycin D (ActD), an inhibitor of RNA polymerase II, both DAZAP1 and hnRNP A1 accumulate in the cytoplasm. The transcription-dependent nuclear localization (TDNL) of hnRNP A1 was first reported two decades ago [11]. Since then, several nucleocytoplasmic shuttling RNA-binding proteins, such as hnRNP K [12], HuR [13], MDDX28 [14], and DAZAP1, have been demonstrated to exhibit this property of nuclear localization. However, the underlying mechanism and the factors involved remain elusive.

Here we report the identification of a signal at the DAZAP1 N-terminus that is both necessary and sufficient for its TDNL. We also found that Steroid receptor RNA activator stem-loop interacting RNA binding protein (SLIRP) interacts with this signal and may be involved in the process.

2. Materials and methods

2.1. Expression vectors

The construction of pDAZAP1-Xpress, its truncation derivatives, and pRed-25 have been previously described [10]. The attachment of segments encoding N-terminal fragments of DAZAP1 to the 5′ end of pRed-25 was carried out by fusion PCR [15]. Site-specific mutagenesis was performed using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The coding region of SLIRP was RT-PCR amplified from RNA isolated from human testis and then cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) for sequence verification. The insert was subsequently cloned into the p3xFLAG-CMV-14 vector (Sigma, St. Louis, MO)

Abbreviations: ActD, actinomycin D; BD, DNA-binding domain; DAZAP1, Deleted in Azoospermia Associated Protein 1; RBD, RNA-binding domain; SLIRP, Steroid receptor RNA activator stem-loop interacting RNA binding protein; TDNL, transcription-dependent nuclear localization.

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http://dx.doi.org/10.1016/j.bbrc.2012.10.076

for the expression of SLIRP-Flag. In addition, the coding region of DAZAP1 was PCR amplified from a *DAZAP1* cDNA clone and inserted in-frame into pEGFP-N1 (Clontech, Palo Alto, CA) to generate the expression vector for DAZAP1-GFP.

2.2. Cell transfection and immunofluorescence staining

The transfection of expression vectors into cultured cells, ActD treatment, and the detection of ectopically expressed proteins were carried out as previously described [10]. GFP and RFP/RED fusion proteins were directly visualized. Epitope-tagged proteins were detected by immunofluorescence staining with primary antibodies against Xpress (Invitrogen) or Flag (Sigma), followed by incubation with Alexa-conjugated secondary antibodies. Confocal microscopy was then used for visualization. Mitochondria were stained with Mitotraker CMXROS (Invitrogen) according to the manufacturer's instruction.

2.3. In vitro RNA binding assay

The binding of DAZAP1 and its truncated derivatives to poly-G and poly-U was carried out as previously described [16]. Briefly, S^{35}-labeled proteins were synthesized *in vitro* and mixed with RNA homopolymers immobilized on agarose beads. Bound proteins were then eluted and analyzed on 8% SDS–polyacrylamide gels, followed by autoradiography.

2.4. Yeast two-hybrid screen

DNA segments encoding N-terminal fragments and intact DAZAP1 were PCR amplified from the human *DAZAP1* cDNA clone and inserted into pAS2-1 (Clontech) to generate the bait plasmids. Over one million transformants of the human testis MATCHMAKER cDNA library in pACT2 (#638810, Clontech) were screened with the bait plasmids as previously described [17].

3. Results and discussion

3.1. Identification of a TDNL signal in DAZAP1

We previously showed that in COS7 cells, exogenously expressed Xpress-tagged DAZAP1 was present predominantly in the nuclei (Fig. 1A) and the nuclear localization of DAZAP1 required the nucleocytoplasmic shuttling signal ZNS at its C-terminus [10]. When the transfected cells were treated with ActD for 3 h, most, if not all, DAZAP1 accumulated in the cytoplasm. To map the region that is required for the TDNL of DAZAP1, we determined the subcellular localization of several truncated DAZAP1 derivatives after ActD treatment. Deletion of the first 52 amino acids in DAZAP1-D3, but not that of the second RBD in DAZAP1-D6, abolished the TDNL of the protein (Fig. 1A). Therefore, the first 52 amino acid residues of DAZAP1 (N52) are required for TDNL. Additional experiments showed that DAZAP1-D3 retained the ability to bind both poly-G and poly-U *in vitro*, which is similar to intact DAZAP1, whereas DAZAP1-D6 could no longer bind poly-U (Fig. S1). The results suggest that TDNL likely does not involve RNA binding.

To test whether N52 is sufficient for TDNL, we linked N52 to the N-terminus of Red-C25, which consists of the red fluorescent protein DsRed2 with the ZNS shuttling signal of DAZAP1 attached to its C-terminus (Fig. 1B). Red-C25 has been previously shown to shuttle between the nucleus and the cytoplasm [10]. While Red-C25 lacked TDNL activity (data not shown), the new fusion protein with N52 (N1-RED) was able to sense the transcriptional activity in the nucleus and remain in the cytoplasm after ActD treatment, indicating that N52 is sufficient for TDNL. Further truncation of N52 from the C-terminal end (in N2-RED) or the N-terminal end (in N3-RED and N4-RED) narrowed the critical region for TDNL down to residues 11–52 (Fig. 1B). This segment, designated N42, therefore contains the signal that is both necessary and sufficient for the TDNL of DAZAP1. N42 consists of the N-terminal portion of the first RBD, and its sequence is highly conserved (>95% identity) among DAZAP1 orthologues in other vertebrates (Fig. S2A). Its similarities with the RBDs of other RNA-binding proteins are much lower (Fig. S2B). The highest scores are found in Musashi homolog 1 (MSI1), hnRNP A1, and hnRNP D, which share less than 60% identity and 80% similarity. There have been no reports thus far on the TDNL of MSI1 or hnRNP D. Whether the N-terminal region of hnRNP A1 also serves as the TDNL signal remains to be determined.

The post-translational modification of proteins plays an important role in the regulation of many cellular processes. Because N42 consists of only 42 amino acid residues, we investigated whether the phosphorylation, methylation, or sumoylation of N42 regulates its function. We identified putative sites for these modifications and changed the residues by site-specific mutagenesis [18] of the Xpress-DAZAP1 expression plasmid (Fig. 2). None of the mutations affected the TDNL of DAZAP1, suggesting that the process may not be regulated by such modifications of the TNDL signal.

3.2. Isolation of N42-interacting proteins

We next screened a human testis yeast two-hybrid library for proteins that interact with the TDNL signal. A bait consisting of intact DAZAP1 linked to the GAL4 DNA-binding domain (BD) did not reveal any clones that had not been previously identified [19]. A bait consisting of N52 turned on the reporter genes by itself without having to interact with target proteins from the library. A deletion of the first 10 amino acid residues of N52 (pAS-N42) resolved the issue of autonomous reporter gene activation. After screening with four-fold coverage of the library, we identified 40 clones that were able to grow on media without histidine and that exhibited β-gal activities, indicating the existence of N42 interaction. Two-thirds of the positive clones, representing at least 3 independent clones, encoded Steroid receptor RNA activator stem-loop interacting RNA binding protein (SLIRP), a ubiquitous small RNA-binding protein with a single RNA recognition motif [20] (Fig. 3). The SLIRP clones lacked the N-terminal portion of the RBD, suggesting that RNA binding is not involved in the interaction.

We confirmed the interaction between N42 and SLIRP using yeast mating assays. Y187 strains carrying various genes fused to the coding sequence for the GAL4-BD in the pAS2-1 vector were mated with Y190 strains carrying various genes fused to the coding sequence of the GAL4 activation domain in the pACT2 vector (Fig. S3A). The ability of the mated diploid cells to grow on plates without histidine (Fig. S3B) and exhibit β-gal activities (Fig. S3C) indicated that SLIRP interacted with N42 (#7, #8), but not ZNS (#9, #10). The DNA-BD encoded by the empty pAS vector (#5, #6) and the human lamin C protein encoded by pLAM5' served as negative controls (#11, #12). The interaction between DAZ and DAZAP1 (#13) served as a positive control.

SLIRP is a ubiquitously expressed small RNA-binding protein that was initially identified in a yeast three-hybrid screen via its binding to the stem-loop structure 7 of Steroid receptor RNA activator (SRA), an RNA activator that mediates the activities of the estrogen receptor [20]. In the nucleus, SLIRP is recruited to estrogen-responsive promoters to repress SRA-mediated coactivation. However, it is present predominantly in the mitochondria, and disruption of the mitochondrial localization sequence at its N-terminus diminishes its ability to repress transcription in the nucleus [20]. SLIRP plays a different role in the mitochondria. It is present in a large ribonucleoprotein complex and maintains mitochondrial

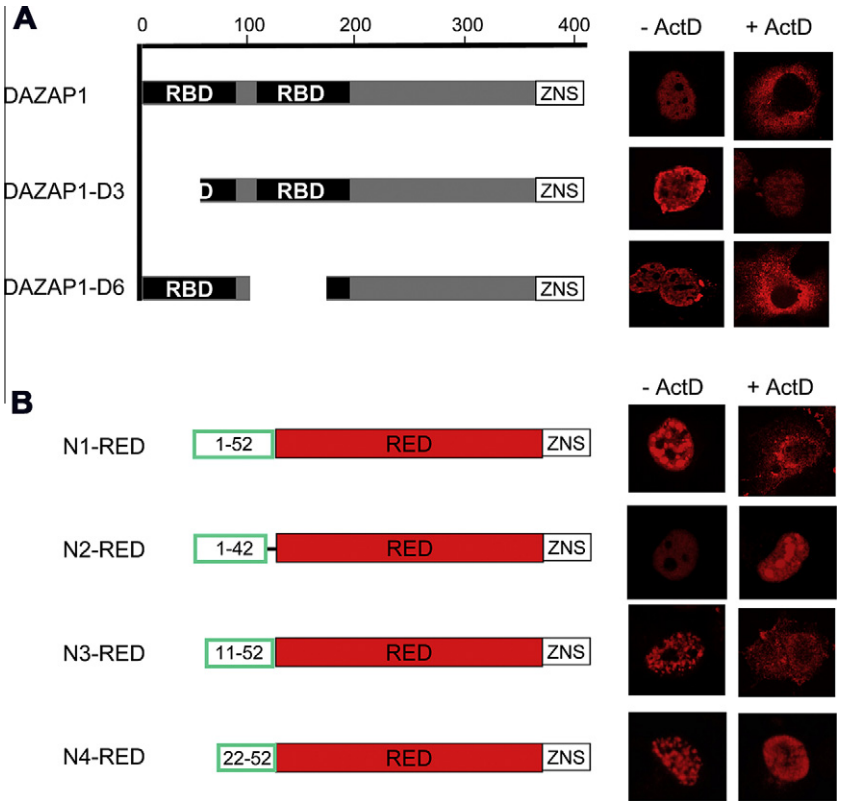

Fig. 1. Identification of the TDNL signal on DAZAP1. (A) Subcellular localization of Xpress-tagged DAZAP1 and its derivatives in COS7 cells transfected with the expression vectors. The cells were treated with or without ActD (5 μg/mL) for 3 h. The proteins were detected by immunostaining with an anti-Xpress antibody. (B) The subcellular localization of DsRed2 (RED) linked to ZNS and various portions of the N-terminal region of DAZAP1. The fluorescence of RED was examined directly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

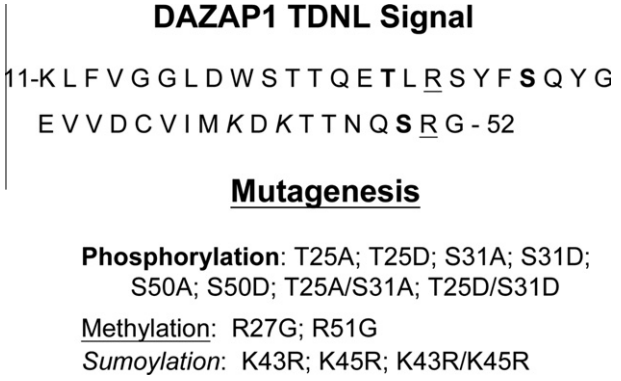

Fig. 2. Mutagenesis of the TDNL signal of DAZAP1. (Top) Amino acid sequence (residues 11–52) of the TDNL signal. Potential sites for phosphorylation (in bold), methylation (underlined) and sumoylation (in italic) are indicated. (Bottom) Amino acid substitutions, generated by site-specific mutagenesis, of potential sites for various post-translational modifications.

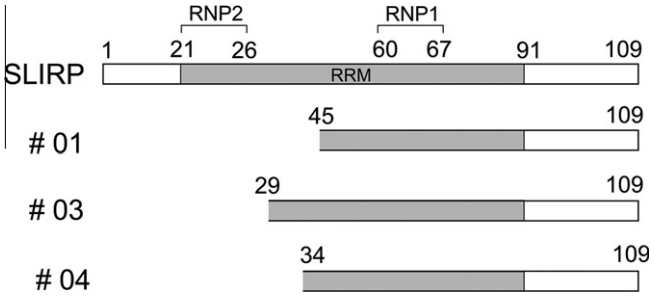

Fig. 3. Structure of the SLIRP protein. The intact protein, consisting of an RNA recognition motif (RRM), is shown at the top. Three yeast two-hybrid clones encoding various lengths of the C-terminal portion are shown below.

mRNA homeostasis by regulating mRNA decay and polyadenylation [21–23]. We confirmed the mitochondrial localization of SLIRP by expressing C-terminal-tagged SLIRP-Flag in HeLa cells (Fig. 4A). Most, if not all, cytoplasmic SLIRP-Flag colocalized with mitochondria, and this localization was not affected by ActD treatment of the cells. Conversely, most endogenous DAZAP1 in ActD-treated cells was retained in the cytoplasm, showing limited mitochondrial localization (Fig. 4B). HeLa cells expressing both SLIRP-Flag and DAZAP1-GFP fusion proteins showed the presence of these two proteins in the mitochondria and nucleus, respectively (Fig. 4C, top panel). In ActD-treated cells, there was significant overlap between the SLIRP-Flag and DAZAP1-GFP signals (Fig. 4C, bottom panel), suggesting that SLIRP overexpression may sequester cytoplasmic DAZAP1 to the mitochondria.

Mitochondrial localization of DAZAP1 is supported by a recent report indicating that the acetylation of the lysine 150 (K150) residue within the second RBD of DAZAP1 regulates its subcellular localization [24]. Sasaki et al. found that both exogenously expressed wild-type DAZAP1 and an acetylation-mimicking K150Q mutant were present almost exclusively in the nuclei of transfected cells, whereas a K150R mutant that could not be acetylated at K150 was present in the cytoplasm and associated with the mitochondria in a large fraction of transfected cells. Using a monoclonal antibody that recognizes an acetylated K150 peptide, the authors found a significant decrease in the level of acetylated K150 in HEK293 cells after ActD treatment, suggesting that DAZAP1 is mostly acetylated in the nucleus rather than in the cytoplasm. Whether K150 acetylation is involved in the NTDL of DAZAP1 remains to be investigated.

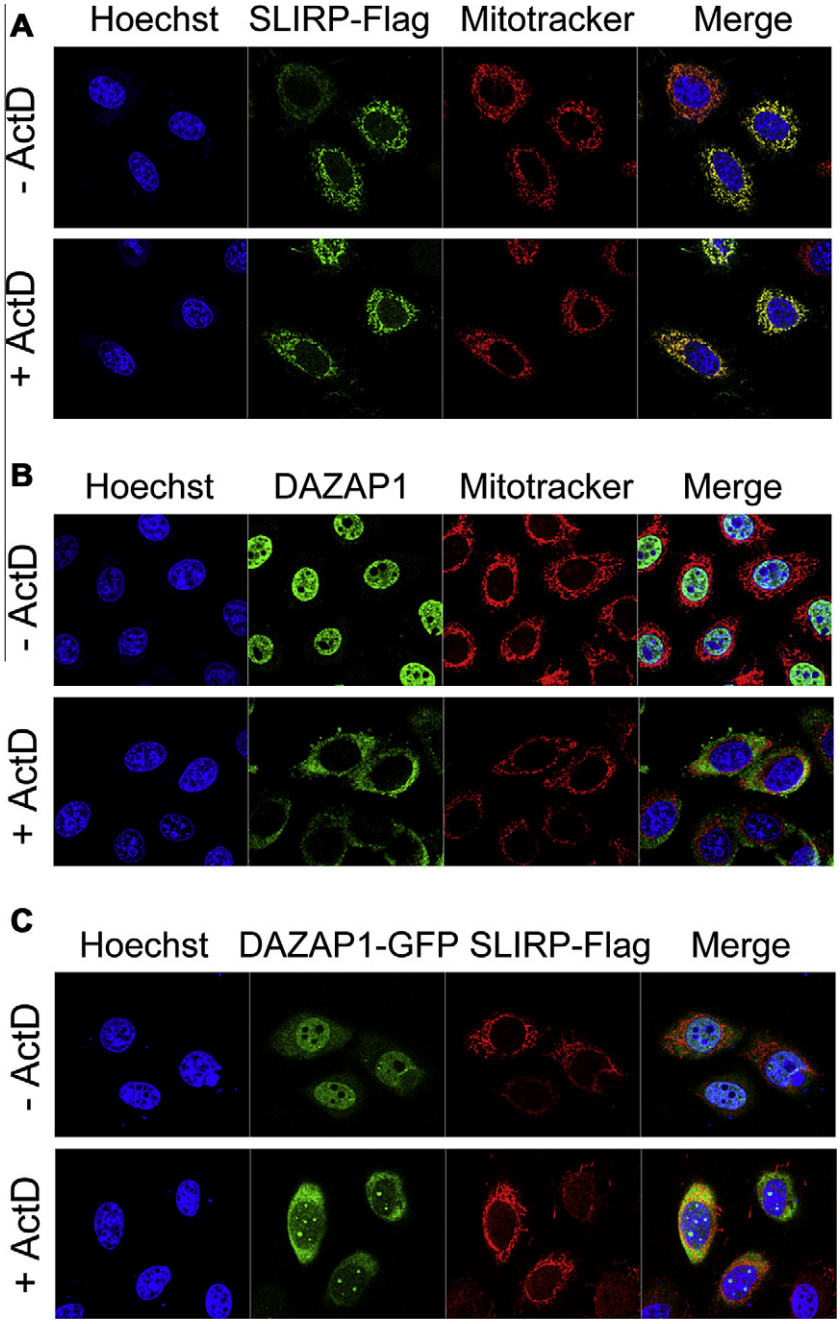

Fig. 4. Subcellular localization of SLIRP and DAZAP1. HeLa cells were transfected with the SLIRP-Flag expression vector alone (A) or together with the DAZAP1-GFP expression vector (C). Mitochondria (in A and B) were stained with Mitotracker CMXROS, and SLIRP-Flag (in A and C) and endogenous DAZAP1 (in B) were detected with antibodies against the Flag epitope and DAZAP1, respectively. The fluorescence of DAZAP1-GFP (in C) was detected directly.

Here we report the identification of a TDNL signal in DAZAP1. Interestingly, a transcription-dependent nuclear export motif (TD-NEM) was recently identified in the von Hippel Lindau tumor suppressor protein (VHL) and a few other proteins [25]. VHL is a component of the VBC-Cul2 E3 ubiquitin ligase complex and is shuttled between the nucleus and the cytoplasm to regulate the activity and stability of the alpha subunit of hypoxia-inducible factor (HIFα) [26]. Unlike DAZAP1, VHL is present predominantly in the cytoplasm but accumulates in the nucleus when RNA polymerase II activity is inhibited by ActD [27]. Khacho et al. first mapped the region required for transcription-dependent nuclear export (TDNE) and found the TD-NEM, which contains the $DXGX_2DX_2L$ sequence. They later identified eEF1A, a cytoplasmic mediator of tRNA export in yeast, as being a TD-NEM binding protein [28]. Subsequent experiments showed that ActD treatment or point mutations within the TD-NEM disrupted the interaction between TD-NEM and eEF1A and resulted in the failure of nuclear export and the accumulation of TD-NEM-containing proteins. Interestingly, eEF1A appears to mediate the nuclear export of these proteins from the cytoplasm without entering the nucleus. The details of this mechanism remain to be elucidated.

Acknowledgments

The work was supported by a grant (NSC 98-2311-B-011-018-MY3) from the National Science Council in Taiwan. We thank Fong-Ping Siao and Yi-Wen Lin for their technical support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.076.

References

[1] S. Tsui, T. Dai, S. Roettger, W. Schempp, E.C. Salido, P.H. Yen, Identification of two novel proteins that interact with germ-cell-specific RNA-binding proteins DAZ and DAZL1, Genomics 65 (2000) 266–273.
[2] E. Goina, N. Skoko, F. Pagani, Binding of DAZAP1 and hnRNPA1/A2 to an exonic splicing silencer in a natural BRCA1 exon 18 mutant, Mol. Cell Biol. 28 (2008) 3850–3860.
[3] T. Pastor, F. Pagani, Interaction of hnRNPA1/A2 and DAZAP1 with an Alu-derived intronic splicing enhancer regulates ATM aberrant splicing, PLoS One 6 (2011) e23349.
[4] N. Skoko, M. Baralle, E. Buratti, F.E. Baralle, The pathological splicing mutation c.6792C>G in NF1 exon 37 causes a change of tenancy between antagonistic splicing factors, FEBS Lett. 582 (2008) 2231–2236.
[5] R.W. Smith, R.C. Anderson, J.W. Smith, M. Brook, W.A. Richardson, N.K. Gray, DAZAP1, an RNA-binding protein required for development and spermatogenesis, can regulate mRNA translation, RNA 17 (2011) 1282–1295.
[6] Y. Vera, T. Dai, A.P. Hikim, Y. Lue, E.C. Salido, R.S. Swerdloff, P.H. Yen, Deleted in azoospermia associated protein 1 shuttles between nucleus and cytoplasm during normal germ cell maturation, J. Androl. 23 (2002) 622–628.
[7] L.C. Hsu, H.Y. Chen, Y.W. Lin, W.C. Chu, M.J. Lin, Y.T. Yan, P.H. Yen, DAZAP1, an hnRNP protein, is required for normal growth and spermatogenesis in mice, RNA 14 (2008) 1814–1822.
[8] G. Dreyfuss, M.J. Matunis, S. Pinol-Roma, C.G. Burd, HnRNP proteins and the biogenesis of mRNA, Annu. Rev. Biochem. 62 (1993) 289–321.
[9] S. Guil, J.F. Caceres, The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a, Nat. Struct. Mol. Biol. 14 (2007) 591–596.
[10] Y.T. Lin, P.H. Yen, A novel nucleocytoplasmic shuttling sequence of DAZAP1, a testis-abundant RNA-binding protein, RNA 12 (2006) 1486–1493.
[11] S. Pinol-Roma, G. Dreyfuss, Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm, Nature 355 (1992) 730–732.
[12] W.M. Michael, P.S. Eder, G. Dreyfuss, The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein, EMBO J. 16 (1997) 3587–3598.
[13] X.C. Fan, J.A. Steitz, HNS, a nuclear-cytoplasmic shuttling sequence in HuR, Proc. Natl. Acad. Sci. USA 95 (1998) 15293–15298.
[14] R. Valgardsdottir, G. Brede, L.G. Eide, E. Frengen, H. Prydz, Cloning and characterization of MDDX28, a putative dead-box helicase with mitochondrial and nuclear localization, J. Biol. Chem. 276 (2001) 32056–32063.
[15] O. Hobert, PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans, Biotechniques 32 (2002) 728–730.
[16] S. Tsui, T. Dai, S.T. Warren, E.C. Salido, P.H. Yen, Association of the mouse infertility factor DAZL1 with actively translating polyribosomes, Biol. Reprod. 62 (2000) 1655–1660.
[17] Y.H. Yu, F.P. Siao, L.C. Hsu, P.H. Yen, TEX11 modulates germ cell proliferation by competing with estrogen receptor beta for the binding to HPIP, Mol. Endocrinol. 26 (2012) 630–642.
[18] R.C. Cadwell, G.F. Joyce, Mutagenic PCR, PCR Methods Appl. 3 (1994) S136–140.
[19] H.T. Yang, M. Peggie, P. Cohen, S. Rousseau, DAZAP1 interacts via its RNA-recognition motifs with the C-termini of other RNA-binding proteins, Biochem. Biophys. Res. Commun. 380 (2009) 705–709.
[20] E.C. Hatchell, S.M. Colley, D.J. Beveridge, M.R. Epis, L.M. Stuart, K.M. Giles, A.D. Redfern, L.E. Miles, A. Barker, L.M. MacDonald, P.G. Arthur, J.C. Lui, J.L. Golding, R.K. McCulloch, C.B. Metcalf, J.A. Wilce, M.C. Wilce, R.B. Lanz, B.W. O'Malley, P.J. Leedman, SLIRP, a small SRA binding protein, is a nuclear receptor corepressor, Mol. Cell 22 (2006) 657–668.
[21] J.M. Baughman, R. Nilsson, V.M. Gohil, D.H. Arlow, Z. Gauhar, V.K. Mootha, A computational screen for regulators of oxidative phosphorylation implicates SLIRP in mitochondrial RNA homeostasis, PLoS Genet. 5 (2009) e1000590.
[22] T. Chujo, T. Ohira, Y. Sakaguchi, N. Goshima, N. Nomura, A. Nagao, T. Suzuki, LRPPRC/SLIRP suppresses PNPase-mediated mRNA decay and promotes polyadenylation in human mitochondria, Nucleic Acids Res. (2012).
[23] F. Sasarman, C. Brunel-Guitton, H. Antonicka, T. Wai, E.A. Shoubridge, LRPPRC and SLIRP interact in a ribonucleoprotein complex that regulates posttranscriptional gene expression in mitochondria, Mol. Biol. Cell 21 (2010) 1315–1323.
[24] K. Sasaki, A. Suzuki, S. Kagatsume, M. Ono, K. Matsuzawa, Y. Taguchi, Y. Kurihara, Acetylation of Prrp K150 regulates the subcellular localization, Gene 491 (2012) 13–19.
[25] M. Khacho, K. Mekhail, K. Pilon-Larose, J. Payette, S. Lee, Cancer-causing mutations in a novel transcription-dependent nuclear export motif of VHL abrogate oxygen-dependent degradation of hypoxia-inducible factor, Mol. Cell. Biol. 28 (2008) 302–314.
[26] P.H. Maxwell, M.S. Wiesener, G.W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher, P.J. Ratcliffe, The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis, Nature 399 (1999) 271–275.
[27] S. Lee, M. Neumann, R. Stearman, R. Stauber, A. Pause, G.N. Pavlakis, R.D. Klausner, Transcription-dependent nuclear-cytoplasmic trafficking is required for the function of the von Hippel-Lindau tumor suppressor protein, Mol. Cell. Biol. 19 (1999) 1486–1497.
[28] M. Khacho, K. Mekhail, K. Pilon-Larose, A. Pause, J. Cote, S. Lee, EEF1A is a novel component of the mammalian nuclear protein export machinery, Mol. Biol. Cell 19 (2008) 5296–5308.