

# Characterization of nucleolar localization and exclusion signals in terminal deoxynucleotidyltransferase interacting factor 2/estrogen receptor -binding protein

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1 **Characterization of nucleolar localization and exclusion signals in terminal**  
2 **deoxynucleotidyltransferase interacting factor 2/estrogen receptor  $\alpha$ -binding**  
3 **protein**

4

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4  
5 **Abstract**

6 Terminal deoxynucleotidyltransferase interacting factor 2/estrogen receptor  $\alpha$ -binding  
7 protein (TdIF2/ERBP) is a multifunctional nucleolar protein. The nucleolar localization  
8 of TdIF2/ERBP is important for its functions because it promotes ribosomal RNA  
9 transcription. However, signal sequences that direct TdIF2/ERBP to the nucleolus are  
10 not well characterized. We examined the TdIF2/ERBP sequence using truncation and  
11 mutation analyses to determine whether the nucleosome binding and C-terminal  
12 domains of TdIF2/ERBP possess nucleolar localization signals (NoLSs). In these  
13 domains, four NoLSs that could direct the mCherry protein to the nucleolus were  
14 detected. In addition, a short stretch of hydrophobic residues (VLLVL) in the center of  
15 TdIF2/ERBP acted as a nucleolar exclusion signal, which reduced the nucleolar  
16 accumulation of mCherry–NoLS fusion proteins. These results would contribute to  
17 improving the prediction of NoLSs from protein sequences. The short, transferrable  
18 localization signals would be valuable tools for understanding the association between  
19 localization and functions of nucleolar proteins.

20  
21 **Abbreviations:** TdIF2: terminal deoxynucleotidyltransferase interacting factor 2;  
22 ERBP: estrogen receptor  $\alpha$ -binding protein; EGFP: enhanced green fluorescent protein;  
23 NLS: nuclear localization signal; NoLS: nucleolar localization signal; NoES: nucleolar  
24 exclusion signal; DAPI: 4',6-diamidino-2-phenylindole

1 **Keywords:** TdIF2; ERBP; nuclear localization signal; nucleolar localization signal;  
2 nucleolar exclusion signal

3

#### 4 **Introduction**

5 The nucleolus, an intranuclear structure lacking a surrounding membrane, is the site of  
6 ribosome assembly and is involved in various cellular processes, such as  
7 ribonucleoprotein assembly, cell proliferation, and stress response [1,2]. A variety of  
8 proteins localize in the nucleolus, many of which dynamically shuttle between the  
9 nucleolus and nucleoplasm or even the cytoplasm [3]. Because the nucleolus is not a  
10 membrane-bound structure, these proteins are considered to accumulate in it via  
11 interactions with nucleolar core components, such as ribosomal DNA; however, detailed  
12 mechanisms underlying their retention remain unclear.

13           Several nucleolar proteins have clusters of basic amino acid residues—  
14 nucleolar localization signals (NoLSs) [3]. These sequences are essential for the  
15 nucleolar localization of proteins, and if the sequences are transferred to unrelated  
16 reporter proteins, they often direct the reporter proteins to the nucleolus. NoLSs  
17 resemble nuclear localization signals (NLSs), and the same sequence often acts as an  
18 NLS and as an NoLS. Scott *et al.* have suggested that it would be useful to divide these  
19 localization signals into three classes: NLS-only, NoLS-only, and joint NoLS–NLS  
20 signals [4]. Although they have reported an artificial neural network (ANN) that can  
21 predict NoLSs, distinguishing between the three classes remains challenging, with the  
22 experimental confirmation of their identity still being necessary.

23           Terminal deoxynucleotidyltransferase interacting factor 2 (TdIF2), also  
24 known as estrogen receptor  $\alpha$ -binding protein (ERBP), is a nucleolar protein that binds  
25 to and inhibits terminal deoxynucleotidyltransferase (TdT) [5,6]. TdIF2/ERBP

1 reportedly promotes ribosomal RNA transcription [7]; therefore, the nucleolar  
2 localization of TdIF2/ERBP is important for its functions. Fujita *et al.* have annotated  
3 residues 631–635 of TdIF2/ERBP as an NLS [5]. Moreover, Koiwai *et al.* have reported  
4 that the N- (1–234) and C-terminal (606–756) fragments of TdIF2/ERBP are sorted to  
5 the nucleolus [7]; however, they have not annotated the exact sequences responsible for  
6 the nucleolar localization of these fragments.

7           In the present study, we examined the TdIF2/ERBP sequence in detail to  
8 identify and characterize its NoLSs. We found that the previously assigned NLS (631–  
9 635) is not necessary for the nucleolar localization of the C-terminal fragment (631–  
10 756). Instead, a short stretch of basic amino acid residues at the C-terminus (750–754)  
11 serves as an NoLS. Two similar clusters of basic residues in the nucleosome binding  
12 domain (NBD) were responsible for the nucleolar localization of the N-terminal  
13 fragment (1–234), and a short cluster of hydrophobic residues (441–445) antagonized  
14 these NoLSs. These results would contribute to improving the prediction of NoLSs from  
15 protein sequences. Moreover, these short, transferrable NoLSs and nucleolar exclusion  
16 signals (NoESs) would serve as valuable tools for controlling the localization of  
17 nucleolar proteins and examining the association between their function and  
18 localization.

19

## 20 **Materials and methods**

### 21 ***Plasmid construction***

22 All oligonucleotides used in the present study are summarized in Table 1. The cDNA  
23 fragment of mCherry was amplified by PCR using a set of primers, mCherry-Forward  
24 and mCherry-Reverse, and pRSET-B-mCherry (provided by Dr. Roger Tsien, Univ.  
25 California, San Diego) as a template. The mammalian expression vector pmCherry-C1

1 was constructed by inserting the *NheI*-*Bgl*II-digested PCR products into the  
2 corresponding sites of the vector backbone of pEGFP-C1 (Clontech, Palo Alto, CA,  
3 USA). The TdIF2/ERBP cDNA was cloned from a cDNA library derived from estrogen-  
4 treated MCF7 cells (OriGene Technologies, Rockville, MD, USA). mCherry-TdIF2 was  
5 constructed by inserting a 2.3-kb fragment of the human TdIF2/ERBP cDNA into the  
6 *Hind*III-*Sal*I sites of pmCherry-C1. The truncated fragments and point mutants of  
7 TdIF2/ERBP were generated using PCR and cloned into pEGFP-C1. Individual nuclear  
8 localization/exclusion signals, namely NLS2 (RVTRRR), NLS3 (RKKPK), NLS4  
9 (KKRRK), NLS6 (RKKKK), and NoES (VLLVL), were chemically synthesized and  
10 annealed (Table 1) and inserted into the *Bgl* II, *Xho* I, *Hind* III, *Sal* I, or *Bam*H I cutting  
11 sites of pmCherry-C1 without additional linkers.

12

### 13 ***Cell culture and transfection***

14 C3H10T1/2 cells were grown in Dulbecco's modified minimum essential medium  
15 containing 10% fetal bovine serum. Plasmid DNA was transfected into the cells using  
16 Lipofectamine LTX and Opti-MEM I (Life Technologies Japan, Tokyo, Japan) in  
17 accordance with the manufacturer's instructions. The transfected cells were incubated at  
18 37°C under 5% CO<sub>2</sub> for 24 h before fluorescence observation.

19

### 20 ***Fluorescence microscopy***

21 Cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10  
22 min and permeabilized using 0.1% Triton X-100 in PBS for 5 min. The cells were  
23 briefly stained using 1-μg/mL DAPI in PBS, following which they were observed using  
24 an Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) and ORCA-ER CCD-  
25 camera (Hamamatsu Photonics, Shizuoka, Japan). Fluorescence intensity was measured

1 by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Table 1

2

### 3 **Results**

#### 4 ***Previously assigned NLS domain is not necessary for the nucleolar localization of the*** 5 ***C-terminal fragment of TdIF2/ERBP***

6 TdIF2/ERBP has been reported to contain four functional domains: NBD, acidic region  
7 (AR), DNA-binding domain (DBD), and NLS domain. Because Koiwai *et al.* have  
8 reported that either the N- (1–234) or C-terminal (606–756) fragment of TdIF2/ERBP  
9 was localized in the nucleolus, there should be multiple NoLSs in the protein. Analysis  
10 of the EGFP-fused deletion mutants revealed that the C-terminal fragment (631–756)  
11 was localized in the nucleolus (Figure 1). Survey of the sequence of this region (631–  
12 756) revealed two NLS-like clusters of basic residues: 631-KKRRK-635 and 750–  
13 RKKKK-754. The NLS domain described by Fujita *et al.* included residues 631–635,  
14 but not 750–754 [5].

Figure 1

15 To examine the role of these clusters in the nucleolar localization of  
16 TdIF2/ERBP, we replaced two basic residues in each of the clusters with alanine  
17 residues. The full-length double mutant EGFP-FL K632A/K633A/K751A/K752A  
18 (Figure 2(a)) was predominantly localized to the nucleoplasm as well as was  
19 accumulated in the nucleolus in a significant proportion of cells (Figure 2(b)),  
20 suggesting that there are additional NLSs and NoLSs in TdIF2/ERBP. To avoid the  
21 effect of the N-terminal fragment, we created EGFP-fused C-terminal fragments (631–  
22 756) with and without the NLS mutations (Figure 2(a)). The EGFP-631–  
23 756/K632A/K633A fragment was localized in the nucleolus, suggesting that the NLS at  
24 631–635 is not indispensable for the nucleolar localization of this fragment (Figure  
25 2(b)). The EGFP-631–756/K751A/K752A and 631–756/K632A/K633A/K751A/K752A

Figure 2

1 fragments showed little accumulation in the nucleolus (Figure 2(b)). These results  
2 suggested that the sequence 750–754 has NoLS activity.

3

4 ***NBD is involved in the nucleolar localization of TdIF2/ERBP but is antagonized by a***  
5 ***nucleolar exclusion signal (NoES)***

6 We created a series of deletion mutants of the N-terminal fragment (1–505) of  
7 TdIF2/ERBP to identify NoLSs in this region (Figure 3). The EGFP-fused fragment 1–  
8 100 was localized in the nucleoplasm, but the fragment 1–133 was accumulated in the  
9 nucleolus, thereby suggesting that NBD is involved in nucleolar localization. Because  
10 the fragment 134–440 was localized in the cytoplasm, the region 1–100 should have an  
11 NLS-only signal(s). Extending the C-terminus of the deletion mutants beyond residue  
12 440 reduced the nucleolar localization of the fragments. The minimum sequence  
13 associated with this antagonizing effect was 441-VLLVL-445, and we named this  
14 hydrophobic sequence the nucleolar exclusion signal (NoES).

15

Figure 3

16 ***TdIF2/ERBP includes at least four NoLSs that direct mCherry protein to the***  
17 ***nucleolus, and NoES partially compromises NoLS activity***

18 To identify more NoLSs, we scanned the full-length TdIF2/ERBP sequence for NLS by  
19 PSORT II [8] because most of the reported NoLSs were rather similar or identical to  
20 NLS consensus sequences. We found six putative NLSs in TdIF2/ERBP (NLS1–6;  
21 Figure 4(a)). NLS4 was a part of the NLS domain reported by Fujita *et al.* [5].  
22 Moreover, we scanned the sequence using NoD ANN developed by Scott *et al.* to  
23 predict NoLSs [4]. NoD predicted four NoLSs, including NLS1, NLS4, NLS5, and  
24 NLS6, in the TdIF2/ERBP sequence.

25 Because NLS1 is included in the fragment 1–100 that was localized in the



1 nucleoplasm (Figure 3), NLS1 is possibly an NLS-only signal rather than an NoLS.  
2 NLS5 resided in the fragment 631–756 that was localized in the nucleoplasm when  
3 NLS4 and 6 were mutated (Figure 2), suggesting that NLS5 is an NLS-only signal. On  
4 this basis, we fused the mCherry gene to synthetic DNA encoding NLS2, NLS3, NLS4,  
5 NLS6, and NoES, and transiently expressed them in C3H10T1/2 cells to observe the  
6 localization of the fusion protein. Furthermore, the fragment 101–134 of TdIF2/ERBP  
7 was used to observe the effect of NLS2, NLS3, and NoES in the endogenous context.  
8 For quantitative analysis, we measured the average fluorescence intensity in the  
9 nucleolus and nucleoplasm and used the ratio (nucleolus/nucleoplasm) as a nucleolar  
10 localization index.

Figure 4

11 All tested NLSs directed mCherry protein to the nucleolus (Figure 4),  
12 suggesting that these sequences are joint NoLS–NLSs that allow the protein to enter the  
13 nucleus and induce accumulation in the nucleolus. On fusing two NLSs to mCherry,  
14 they synergistically enhanced the nucleolar localization of the fusion protein. Although  
15 the addition of NoES reduced this nucleolar localization (Figure 5), most of the fusion  
16 protein was retained in the nucleus (Figure 4(b)).

Figure 5

## 18 Discussion

19 In the present study, we examined the TdIF2/ERBP protein sequence and found four  
20 NoLSs (NLS2, NLS3, NLS4, and NLS6) and one NoES. Our findings demonstrated  
21 that NLS1 and NLS5 are possibly NLS-only signals rather than NoLSs. The relative  
22 activity of NoLSs to direct mCherry protein to the nucleolus appeared to be in the order  
23 NLS3 and NLS6 > NLS2 and NLS4, as measured by the fluorescence intensity ratio  
24 (nucleolus/nucleoplasm). Notably, NLS4, which was the only NLS annotated on  
25 TdIF2/ERBP by Fujita *et al.* [5], was not necessary for the nucleolar localization of the

1 C-terminal fragment (631–756) provided that NLS6 was intact, suggesting that NLS4 is  
2 a relatively weak NoLS.

3 No simple consensus sequence is known for NoLS, but Scott *et al.* have  
4 reported that an ANN can predict NoLSs from protein sequences [4]. Their ANN  
5 predicted four putative NoLSs—NLS1, NLS4, NLS5, and NLS6—in the TdIF2/ERBP  
6 sequence. However, our results suggested that NLS1 and NLS5 are possibly NLS-only  
7 signals, whereas NLS2 and NLS3 are NoLS. These discrepancies indicate that  
8 distinguishing between NoLS and NLS-only signals without experimental analysis  
9 remains challenging.

10 Martin *et al.* have reported that arginine hexamer peptide, but not lysine  
11 hexamer, was localized to the nucleolus. They found that if another NLS existed nearby,  
12 four arginine residues were sufficient for the nucleolar localization of GFP [9]. They  
13 have concluded that the high isoelectric point ( $pI > 12.6$ ) and the distribution pattern of  
14 the positive charge on the molecular surface of the NLS domain were important to  
15 distinguish between NoLS and NLS-only signals. Although the cumulative effect of  
16 NoLSs (Figure 4) would support their conclusion, it is not the sole principle of the  
17 nucleolar localization of protein because the individual NoLS of TdIF2/ERBP could  
18 localize mCherry protein to the nucleolus without extra NLS. Further research is  
19 required to resolve this issue.

20 In the center of the TdIF2/ERBP protein, we found an NoES that reduced the  
21 nucleolar accumulation of the protein. Unlike the nuclear export signal (NES), NoES  
22 showed little effect on the nucleoplasmic localization of TdIF2/SRBP. Meng *et al.* have  
23 reported similar signals in two nucleolar proteins, GNL3L and Ngp1, and named them  
24 nucleoplasmic localization signals (NpLSs) [10]. However, they assigned NpLS activity  
25 to relatively large domains (285 and 272 residues for GNL3L and Ngp1, respectively),

1 whereas the minimum sequence of NoES was only five residues. We found no NoES-  
2 like sequence (*i.e.*, five successive hydrophobic residues) in the reported NpLSs. These  
3 findings suggest that NoES and NpLS are functionally similar but structurally different  
4 signals. The existence of functionally similar signals in multiple nucleolar proteins  
5 suggests the importance of these NoLS-antagonizing signals in regulating the  
6 localization and function of nucleolar proteins.

7           The leucine-rich feature of the NoES may suggest a relationship to the  
8 leucine-rich NESs. Leucine-rich NESs are well characterized and mostly comprise one  
9 of the three consensus sequences: [ $\Phi$ -X<sub>2,3</sub>- $\Phi$ -X<sub>2,3</sub>- $\Phi$ -X- $\Phi$ ] (class 1), [ $\Phi$ -X- $\Phi$ -X<sub>2</sub>- $\Phi$ -  
10 X- $\Phi$ ] (class 2), and [ $\Phi$ -X<sub>2</sub>- $\Phi$ -X<sub>3</sub>- $\Phi$ -X<sub>2</sub>- $\Phi$ ] (class 3), where  $\Phi$  represents large  
11 hydrophobic residues and X<sub>2,3</sub> any two or three amino acids [11]. However, the NoES  
12 sequence (VLLVL) does not conform to any of these consensus sequence classes  
13 because it does not have sufficient spacing between the hydrophobic residues.  
14 Moreover, an NESmapper [11] scan of the TdIF2/ERBP sequence failed to identify the  
15 NoES as a NES. These facts suggest that the NoES is not a variant of leucine-rich NES,  
16 although we cannot completely dismiss the possibility that the NoES works as a weak  
17 NES.

18           In summary, we found four NoLSs and one NoES in TdIF2/ERBP. Our  
19 findings will contribute to improving the prediction of NoLSs from protein sequences.  
20 Because the NoES is only five residues long, it could easily be inserted into other  
21 nucleolar proteins with the expectation of reducing their accumulation in the nucleolus.  
22 This method would be valuable for studying the association between the localization  
23 and function of nucleolar proteins.

24

25

1 **Author contribution**

2 Kenji Sugimoto designed the research. Shun Shibata, Toshihiro Ueda, Katsuhiko Sasaki,  
3 Yukiko Shimoida, and Kaori Senda-Murata performed the experiments and analyzed  
4 data. Takshi Fukada analyzed data and wrote the manuscript.

5

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9

1 **Figure legends**

2

3 **Figure 1.** Subcellular localization of the C-terminal fragments of TdIF2/ERBP. (a)  
4 Schematic diagram of truncated fragments of TdIF2/ERBP. NBD: nucleosome binding  
5 domain; AR: acidic region; DBD: DNA-binding domain; NLS: nuclear localization  
6 signal. (b) The truncated fragments of TdIF2/ERBP were fused with EGFP, transiently  
7 expressed in C3H10T1/2 cells, and observed using fluorescence microscopy. We  
8 observed at least 50 cells for each fragment and the localization pattern in all the cells  
9 was similar to those shown.

10

11 **Figure 2.** Subcellular localization of TdIF2/ERBP fragments with NLS mutations. (a)  
12 Schematic diagram of potential NLSs in the C-terminal domain of TdIF2/ERBP and  
13 mutant fragments. Solid boxes: wildtype NLS; open boxes: mutated NLS. (b) Wildtype  
14 or mutant fragments of TdIF2/ERBP were fused with EGFP, transiently expressed in  
15 C3H10T1/2 cells, and observed using fluorescence microscopy. We observed at least 50  
16 cells for each fragment and the localization pattern in all the cells was similar to those  
17 shown. However, because the full-length double mutant (full-length  
18 K632A/K633A/K751A/K752A) showed two different localization patterns (nucleolus  
19 or nucleoplasm), the proportion of cells showing each pattern is indicated.

20

21 **Figure 3.** Subcellular localization of the N-terminal fragments of TdIF2/ERBP. (a)  
22 Schematic diagram of truncated fragments of TdIF2/ERBP and the proportions of cells  
23 that showed the nucleolar, nucleoplasmic, and cytoplasmic localization of the EGFP-  
24 fused fragments in C3H10T1/2 cells. No: nucleolus; Nu: nucleoplasm; Cyt: cytoplasm.  
25 (b) Representative cell images showing EGFP-fused N-terminal fragments of

1 TdIF2/ERBP.

2

3 **Figure 4.** Effects of the putative NLSs and NoES on mCherry protein localization. (a)

4 Locations and sequences of the putative NLSs and NoES in TdIF2/ERBP. Basic

5 residues are underlined. (b) Schematic drawing showing the structures of mCherry-

6 fused NLSs and NoES. mCherry-fused TdIF2/ERBP fragment (101–134), which

7 includes NLS2 and NLS3, is shown. The numbers indicate the proportions of cells that

8 showed the nucleolar localization of the mCherry fusion proteins, and the ratios of

9 average fluorescence intensities (nucleolus/nucleoplasm) in C3H10T1/2 cells.

10 Moreover, representative cell images indicating the localization of mCherry fusion

11 proteins are shown. For each construct, we observed at least 50 cells for the cell

12 numbers (%), and analyzed 10 cells for fluorescence intensity.

13

14 **Figure 5.** Antagonistic activity of the NoES to the nucleolar accumulation of mCherry–

15 NoLS fusion proteins. Ratios of average fluorescence intensities

16 (nucleolus/nucleoplasm) in C3H10T1/2 cells are indicated for the fusion proteins with

17 (blank bars) NoES or without (solid bars) NoES. Error bars: standard deviation (n = 10).