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3′-Untranslated region of *doublecortin* mRNA is a binding target of the Musashi1 RNA-binding protein

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ABSTRACT

Musashi1 (Msi1) is an RNA-binding protein that is highly expressed in neural stem cells, and is considered to be a stemness factor. A known function of Msi1 is translational repression of specifically bound mRNAs. Although the basic mechanism and some target RNAs have been reported, further survey of interactors is necessary to understand the integrated function of Msi1. By screening using an mRNA display technique, we found that *doublecortin* (*dcx*) mRNA is a specific binding target of Msi1 in vitro. We confirmed that Msil repressed translation of a luciferase reporter gene linked to the selected 3′-untranslated region fragment of *dcx* in Neuro2A cells.

1. Introduction

The Musashi family is a conserved group of RNA-binding proteins. In mammalian nervous system, Musashi1 (Msi1) is expressed in neural precursors [1], including neural stem cells (NSCs), and is considered to be a key molecule for stemness [2]. It contributes to maintenance of the stem cell state of NSCs by binding to the 3′-untranslated region (UTR) of target mRNAs, *m-numb* and $p21^{WAF\text{-}1}$, and repressing their translation [3,4]. *m-numb* and $p21^{WAF\text{-}1}$ encode an inhibitor of Notch signaling, and a cyclin-dependent kinase inhibitor, respectively. In *Drosophila*, Msi1 also represses translation of a transcriptional repressor, *ttk69*, and controls asymmetric cell division of a sensory organ [5]. The mechanism of the translational repression by Msi1 was recently clarified in detail, *i.e.*, Msi1 competitively inhibits interaction between poly(A)-binding protein (PABP) and eukaryotic translation initiation factor 4G (eIF4G), and thereby blocks translation [6]. Although the basic molecular mechanism and some important target mRNAs have already been reported, further survey of up- and down-stream interactors of Msi1 is necessary for a comprehensive understanding of its role in NSCs.

In this study, we tried to screen proteins and mRNAs interacting with Msi1 by using an mRNA display technique, named in vitro virus (IVV), which employs a library of protein (phenotype)-mRNA (genotype) linked molecules [7], and was originally used to screen functional proteins from cDNA libraries [8]. We thought that target mRNAs of Msi1 might be screened simultaneously with target proteins in a genomic- systematic evolution of ligands by exponential enrichment (SELEX)-like fashion (Fig. 1) [9].

The screening yielded three candidate target mRNAs of Msi1, including a 3′UTR fragment of *doublecortin* (*dcx*) mRNA (Fig. 2A), which is a gene related to migration of newborn neurons and neural development [10,11]. We confirmed specific interaction between Msi1 and *dcx* mRNA in vitro, and demonstrated a functional relation between the genes using reporter gene assay.

2. Materials and methods

2.1. Msi1, hnRNP K, and mock proteins

Plasmids encoding full-length mouse Msi1, heterogeneous nuclear ribonucleoprotein K (hnRNP K) and a mock construct

Abbreviations: Msi1, Musashi1; NSCs, neural stem cells; UTR, untranslated region; WAF-1, wild-type p53-activated fragment 1; PABP, poly(A)-binding protein; eIF4G, eukaryotic translation initiation factor 4G; IVV, in vitro virus; SELEX, systematic evolution of ligands by exponential enrichment; Dcx, doublecortin; hnRNP K, heterogeneous nuclear ribonucleoprotein K; TAP, tandem affinity purification; RIP, RNA immunoprecipitation; MCS, multi-cloning site; RT-PCR, reverse transcription-polymerase chain reaction; Chr., chromosome

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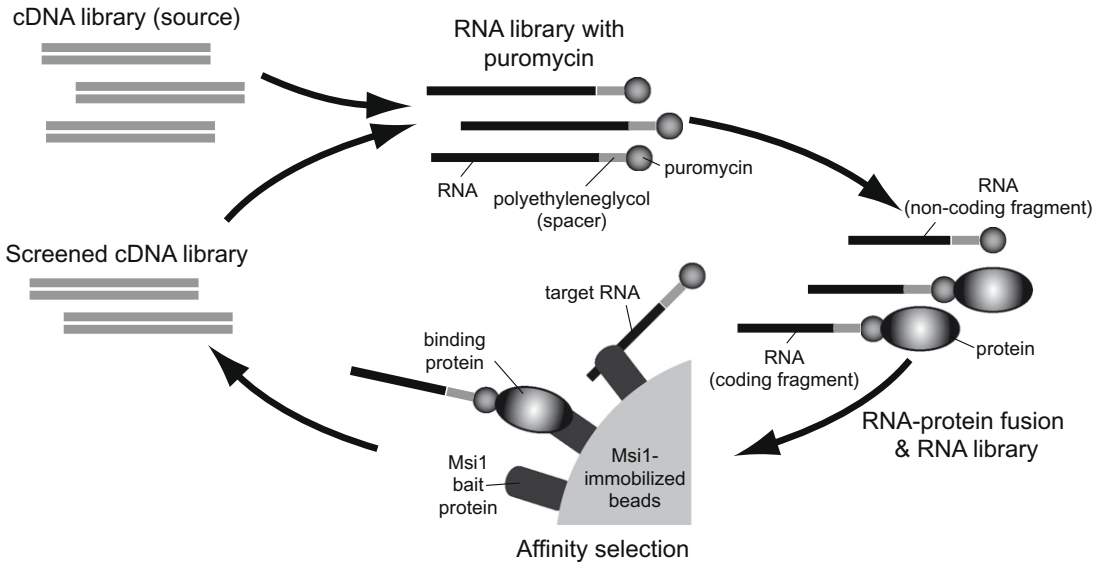

Fig. 1. Schematic representation of an mRNA display screening. A bait protein fused with affinity-tags was co-translated with a template RNA library for mRNA display in a wheat germ extract. The library was prepared from poly(A)+ RNA extracted from E12 mouse embryonic brain as described previously [3]. Complexes of the bait and targets were captured by affinity beads. The recovered mRNAs and proteins displaying mRNA were amplified by RT-PCR, and in vitro transcribed. After iterative screening, the selected cDNAs were cloned and sequenced.

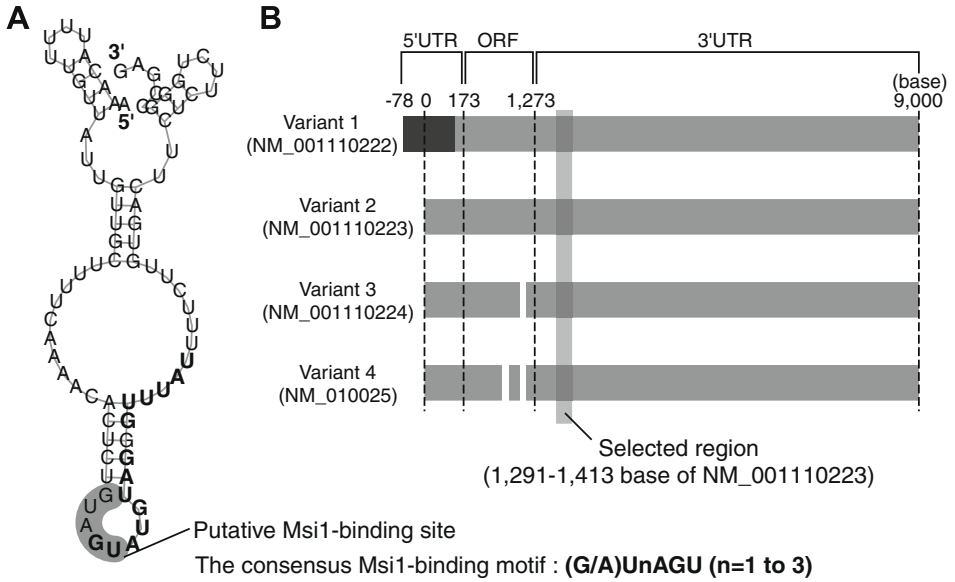

Fig. 2. Predicted 2D structure of the selected region and full-length mRNAs of *dcx*. (A) Predicted structure of the selected region of *dcx* RNA. The shaded region and the boldface letters indicate an Msi1-recognizing motif and similar sequences, respectively. (B) The mRNA structures of four variants of *dcx*. The 3′UTR sequences are identical.

containing T7- and tandem affinity purification (TAP)-tags (Fig. 3A) were prepared as described previously [8]. For expression in cultured cells, the cells were transfected with these plasmids. For in vitro expression using a wheat germ extract cell-free translation system (Zoegene), templates for the proteins were PCR-amplified with primers (Supplementary Table S1), transcribed with a RiboMAX RNA production system-SP6 (Promega), and purified with an RNeasy Mini kit (Qiagen).

2.2. Screening and sequence analysis

Preparation of Msi1 bait protein and mRNA-displayed protein libraries, and the procedure of the IVV screening method were described in detail in our previous report [8]. After screening, artificial sequences were removed from the selected clones, and BLASTn search was carried out. The sequences were also grouped into clusters using CLUSTALW.

2.3. Prediction of 2D structure of RNAs

Representative RNA sequences of each cluster having averaged length were subjected to 2D structure prediction by RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

2.4. In vitro pull-down assay

Pull-down assay was performed as described previously [3] with some modifications. In brief, fragments of *dcx* (1332–1431 base region of NM_001110223), antisense *dcx*, and mutated *dcx* (all U and G residues were converted to A and C, respectively) were

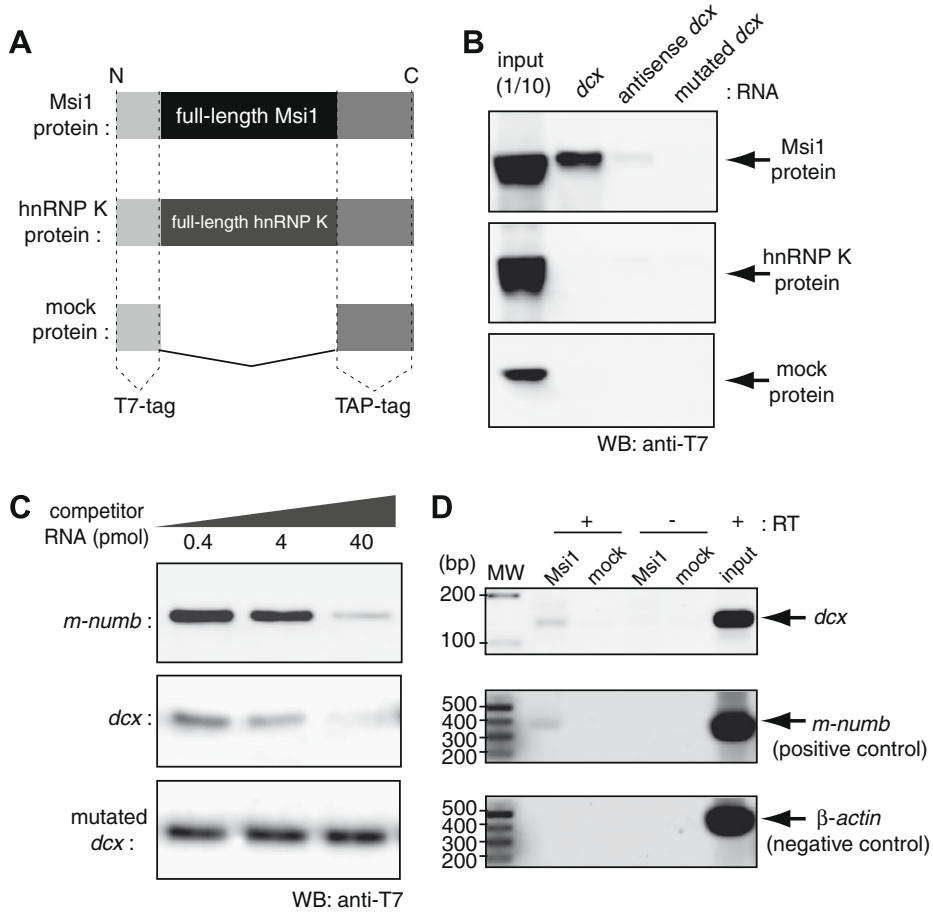

Fig. 3. In vitro binding of Msi1 to *dcx* RNA and RIP assay. (A) Schematic diagrams of Msi1, hnRNP K, and mock protein. (B) In vitro binding assay between immobilized *dcx*, antisense *dcx*, or mutated *dcx* RNAs, and Msi1, hnRNP K, or mock proteins. (C) In vitro binding assay with various amounts of the competitor RNA, *m-numb*, *dcx*, or mutated *dcx*. (D) RNA-binding assay combining affinity precipitation with RT-PCR. *m-numb* and *β-actin* mRNAs were positive and negative controls, respectively.

PCR-amplified and modified from a clone, transcribed by using a MEGAscript SP6 kit (Ambion) with biotin-14-CTP (Invitrogen), immobilized on MAGNOTEX-SA beads (Takara), and incubated with in vitro-translated proteins. The bound proteins were collected and detected by Western blotting with anti-T7 mAb (Novagen).

2.5. Competitive inhibition assay

The procedure was basically the same as described above for pull-down assay, except for addition of biotin-unlabeled competitor RNAs, *i.e.*, a known Msi1-binding region of *m-numb* RNA (2863–2945 base region of BC033459), a *dcx* RNA fragment, and a mutated *dcx* RNA. The *m-numb* RNA was cloned from the starting library for screening by PCR using specific primers (Supplementary Table S1), and transcribed with the RiboMAX kit.

2.6. RNA immunoprecipitation (RIP) assay

RIP assay was performed in Neuro2A cells as described previously [3] with some modifications. After immunoprecipitation with rabbit IgG agarose (SIGMA), reverse transcription-polymerase chain reaction (RT-PCR) experiments were performed with specific primers for *dcx*, *m-numb*, and *β-actin* (Supplementary Table S1). An input RNA sample was also assessed by RT-PCR. The numbers of PCR cycles for *dcx*, *m-numb*, and *β-actin* were 36, 30, and 20, respectively.

2.7. Reporter gene assay

To construct a reporter gene vector, a fragment of the 3′UTR of *dcx* (1247–2028 base region of NM001110223; DNAFORM) was sub-cloned into a multi-cloning site (MCS; 5′-CTAGACGCGT-GAATTCTTAAGCATATGTCGCGACCTAG-3′) joined to the 3′UTR of *luciferase* in pGL4.13 (Invitrogen). A pGL4.13 vector containing mutated 3′UTR of *dcx* was also constructed (the mutation was the same as described in Section 2.3). The pCMV-T7-Msi1-TAP and pCMV-T7-TAP constructs were used as effectors. Neuro2A cells in 24-well plates were co-transfected with reporter genes (100 ng), effector genes (360 ng), and control pRL-TK plasmid (40 ng; Promega). After 2 days of incubation, the reporter activities were measured using a Dual-Luciferase reporter assay system (Promega).

2.8. Real-time RT-PCR

Total RNAs of Neuro2A cells, which had been transfected as described above, were collected using an RNeasy kit. The RNA samples (10 ng each) were employed for quantification of the transfected reporter gene mRNAs using a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and LightCycler (Roche) with specific primers (Supplementary Table S1). The relative expression of each RNA was calculated by means of the ΔΔCt method as follows: (1) ΔCt = mRNA (*firefly luciferase*) − mRNA (*renilla luciferase*); (2) ΔΔCt = ΔCt (target sample) − ΔCt (control sample); (3) relative expression value = 2^(−ΔΔCt).

3. Results

3.1. mRNA fragments screened as binding targets of Msi1

To identify mRNAs and proteins specifically interacting with Msi1, we performed in vitro affinity selection from a cDNA library of E12 embryonic mouse brain using the IVV method (Fig. 1). After 6 rounds of iterative screening, we obtained sequences of 128 selected clones. Among them, 83 (65%) were clustered into 14 distinct sequence groups (Table 1), and others consisted of single clones. We considered that the 14 multiple sequence groups are candidates for interactors with Msi1, because specific binders should be enriched during such screening [8]. The clones in each group were basically identical, except for some extensions at the 5′- or 3′-ends, so we choose minimal length clones as representatives of each group for subsequent experiments. The results of a BLAST search suggests that all of the candidates might bind to Msi1 as RNA molecules, because all of the candidates were found to be non-coding sequences and to have known Msi1-recognized motifs or similar sequences in the selected regions (Table 1; Supplementary Figs. 1–7) [3]. Among the 14 candidates, 11 were not aligned to any mRNA sequence or were chimeric artifacts, and therefore we concluded that these were false-positives. Of the remaining three, *dcx*, *ubcM2*, and *gdi1*, we focused on *dcx*, because the sequence has characteristics consistent with those mentioned in previous reports [3,4,6], namely: (1) The sequence includes an Msi1-binding motif and three tandem similar sequences (Fig. 2A). (2) Most of these motifs are located near or on the putative stem-loop structure (Fig. 2A). (3) The selected region is located on the 3′UTR of the mRNA (Fig. 2B). Thus, we further analyzed the *dcx* mRNA.

3.2. Msi1 bound specifically to the 3′UTR of dcx mRNA in vitro

To confirm that the selected *dcx* mRNA is an interactor of Msi1, we performed in vitro binding assay between Msi1 protein (Fig. 3A) and the selected region of the *dcx* mRNA, with several controls (Fig. 2A). Msi1 bound only to the *dcx* mRNA fragment. hnRNP K, which has another RNA-binding specificity [6,12], and mock proteins (Fig. 3A) did not bind to any of the RNAs tested (Fig. 3B).

Next, we performed a competitive binding assay between Msi1 protein and *dcx* mRNA in the presence of RNA fragments of *m-numb* (a known Msi1-interacting mRNA), a non-biotinized *dcx* (positive control), or a mutated *dcx* (negative control). The *m-numb* RNA decreased the binding efficiency between immobilized *dcx* RNA and Msi1 in a dose-dependent fashion (Fig. 3C), as did the non-biotinized *dcx*, but the mutated *dcx* had no effect. These results suggest that *dcx* RNA binds specifically to Msi1 protein in a similar fashion to *m-numb* mRNA.

3.3. Exogenous Msi1 protein interacted with endogenous dcx mRNA in cultured cells

Furthermore, we examined the binding between Msi1 and *dcx* mRNA in cultured cells, employing a RIP assay. We precipitated an exogenous Msi1-endogenous RNA complex from Neuro2A cells that had been transfected with an expression vector for Msi1 or a mock protein (Fig. 3A). After the precipitation, the co-precipitated mRNAs were amplified by RT-PCR with specific primers for *dcx*, *m-numb*, and *β-actin*. RNAs that bound to the Msi1 protein gave rise to an RT-PCR product when the *dcx* and *m-numb* primers, but not the *β-actin* primers, were used (Fig. 3D). On the other hand, the mock protein failed to show binding to any mRNAs. These results demonstrate that Msi1 can specifically interact with endogenous *dcx* mRNA, as well as *m-numb* mRNA in cultured cells.

3.4. Msi1 suppressed translation of a reporter gene coupled to 3′UTR region of dcx

To investigate whether the selected region of the *dcx* mRNA can mediate translational repression, we used a reporter assay system. We transiently co-transfected a plasmid encoding *firefly luciferase* reporter linked on the downstream side with the 3′UTR region of *dcx* and the Msi1 expression plasmid into Neuro2A cells. Although the Neuro2A cells express endogenous Msi1 protein, its expression level was extremely low as compared with that of transfected Msi1 (Supplementary Fig. 8A), and the protein has little or no effect on the reporter expression (Supplementary Fig. 8B). The decrease of luciferase enzymatic activity after Msi1 expression was obtained by comparing the construct containing the 3′UTR of *dcx* mRNA with a control reporter construct. As shown in Fig. 4A, the level of luciferase enzymatic activity was significantly reduced (∼34%) specifically in the cells co-transfected with both plasmids.

Furthermore, RNA quantification using real-time RT-PCR showed that Msi1 did not affect the relative amount of reporter gene mRNA (Fig. 4B), indicating that Msi1 acted by repressing translation, rather than by altering the steady-state RNA level.

4. Discussion

We performed screening for interactors of Msi1, *i.e.,* co-factor proteins and target mRNAs, because the molecular mechanism of

Table 1
The selected candidates from mRNA display screening.

Description	Accession no.	Number of clones	Average length (bp)	Selected region (bp)	Msi1-binding motif[a]
3′UTR of doublecortin (Dcx) mRNA	NM_010025.2	35	101	1291–1413	1
Chr.11 genomic contig[b]	NW_001030424.1	9	147	20 458 673–20 459 030	16
Mitochondrian genome[b]	NC_005089.1	8	108	12 963–13 045	3
Chr.16 genomic contig	NT_039625.7	6	116	29 556 467–29 556 628	5
Chr.4 genomic contig	NT_109317.2	4	169	6 902 491–6 902 662	1
Chr.3 genomic contig[b]	NT_039240.7	4	204	34 003 994–34 004 276	20
3′UTR of leucyl-tRNA synthetase (Lars2) mRNA[b]	NM_153168.2	3	136	3339–3387	0
Chr.3 genomic contig	NT_162143.3	2	111	4 251 697–4 251 802	1
3′UTR similar to that of ubiquitin-conjugating enzyme (UbcM2) mRNA	XM_001477380.1	2	278	1145–1420	0
Chr.1 genomic contig	NT_039170.7	2	132	52 173 355–52 173 490	13
5′UTR of GDP dissociation inhibitor 1 (Gdi1) mRNA	NM_010273.2	2	67	33–109	2
Chr.18 genomic contig[b]	NT_039674.7	2	141	43 917 837–43 917 939	2
Not identified	–	2	96	–	3
Chr.1 genomic contig	NT_039185.7	2	158	9 800 874–9 801 028	1

[a] Number of Msi1-binding motifs in the selected sequences.
[b] Chimeric artifacts.

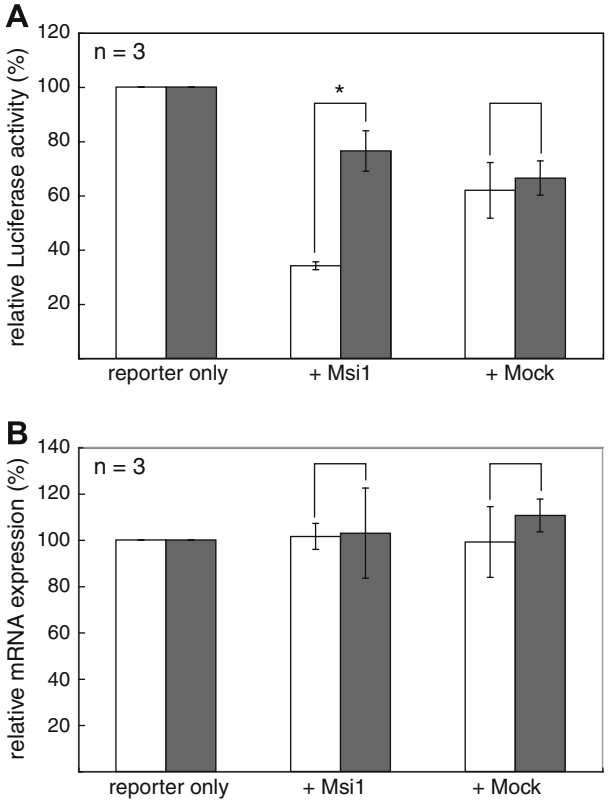

Fig. 4. Post-translational down-regulation of a reporter gene by Msi1 and 3′UTR of *dcx*. (A) Luciferase reporter assay. White and gray bars indicate the relative luciferase activity (*firefly luciferase*/*renilla luciferase*) of a reporter construct with the 3′UTR region of *dcx* and mutated *dcx*, respectively. $^{*}P < 0.05$. (B) Relative level of reporter mRNAs. White and gray bars indicate the relative transcript levels of a reporter gene coupled with the 3′UTR region of *dcx* and mutated *dcx*, respectively.

Msi1 in translational repression, such as inhibition of ribosome formation, is not fully understood [6]. Moreover, comprehensive screening for target mRNAs focused on the stemness of NSCs has not been reported, though a RIP-chip assay for Msi1 using HEK293T cells has been done [13]. In the screening, we had expected to find both protein and mRNA interactors, but in fact only non-coding RNAs were identified (Table 1). We did not detect specific enrichment of any protein interactors, including a known Msi1-binding protein, PABP (data not shown). This might have been because the affinity between Msi1 and target RNA is high (dissociation constant 4 nM) [3], and this might prevent enrichment of Msi1-binding proteins in the screening process. In order to select protein binders of Msi1, a suitable strategy might be to introduce into the bait protein mutations that would inhibit RNA binding [3].

We obtained three candidate mRNAs, *dcx*, *ubcM2*, and *gdi1*, encoding a microtubule-associated protein, an ubiquitin-conjugating enzyme, and a GTPase activator, respectively. Among them, we focused on *dcx*, because the mRNA has not only structural features, *i.e.,* Msi1-binding motif and putative stem-loop structure in the 3′UTR, but also functional characters associated with the *msi1* gene as follows. (1) Dcx protein is expressed only in neuronal precursors just differentiated from NSCs [14]. (2) Mutually exclusive 'protein' expression of Msi1 and Dcx in human brain was observed [15]. (3) Knock-out of Musashi family genes reduced the number of neurospheres isolated from embryonic mouse brains [16], while knock-down of *dcx* prevented migration of cells from neurospheres and their structure was maintained [17]. These findings imply that the Msi1 protein may bind to, and inhibit translation of *dcx* mRNA, thereby preventing inappropriate migration of NSCs in vivo. Although we demonstrated that Msi1 binds to *dcx* mRNA in vitro (Fig. 3B and C) and in cultured cells (Fig. 3D), and also suppresses translation of a reporter gene coupled to the *dcx* 3′UTR region (Fig. 4), further studies to confirm the binding of the endogenous molecules and the repression of Dcx translation in vivo are imperative. However, expression of the *dcx* mRNA in neuronal-derived cell lines, Neuro2A, P19C6, and SK-N-SH, is extremely low or below the limit of detection (data not shown). Thus, studies employing NSCs in vivo or ex vivo are necessary to obtain direct evidence of functional linkage between Msi1 and *dcx* mRNA in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.06.045.

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