Identification of Nuclear Localization Signals in the Human Homeoprotein, MSX1

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18

19 **Abstract**

20 MSX1 is one of the homeoproteins with the homeodomain (HD) sequence, which regulates
 21 proliferation and differentiation of mesenchymal cells. In this study, we investigated the
 22 nuclear localization signal (NLS) in the MSX1 HD by deletion and amino acid substitution
 23 analyses. The web-based tool NLStradamus predicted two putative basic motifs in the N- and
 24 C-termini of the MSX1 HD. Green fluorescent protein (GFP) chimera studies revealed that
 25 NLS1 (₁₆₁RKHKTNRKPR₁₇₀) and NLS2 (₂₁₆NRRAKAKR₂₂₃) were independently
 26 insufficient for robust nuclear localization. However, they can work cooperatively to promote
 27 nuclear localization of MSX1, as was shown by the two tandem NLS motifs partially
 28 restoring functional NLS, leading to a significant nuclear accumulation of the GFP chimera.
 29 These results demonstrate a unique NLS motif in MSX1, which consists of an essential single
 30 core motif in helix-I, with weak potency, and an auxiliary subdomain in helix-III, which alone
 31 does not have nuclear localization potency. Additionally, other peptide sequences, other than
 32 predicted two motifs in the spacer, may be necessary for complete nuclear localization in
 33 MSX1 HD.

34 **Keywords;** MSX1, homeodomain (HD), nuclear localization signal (NLS)

35

36 **Introduction**

37 Homeoproteins are transcription factors that regulate cell proliferation and differentiation
38 Ladam and Sagerstrom 2014. These proteins contain a highly conserved polypeptide, termed
39 the homeodomain (HD). This domain enables them to interact with target DNA sequences,
40 and also several other proteins, to regulate various biological processes Zhang et al. 1996; Ye
41 et al. 2011; Hovde et al. 2001. Homo- and heterodimeric protein–protein interactions are
42 considered particularly important for regulating the actions of HD-containing transcriptional
43 regulatory proteins, including Msx homeoprotein families Zhang et al. 1996; Zhang et al.
44 1997. The expression of *MSX1* is tightly controlled, both spatially and temporally, during
45 development as this gene regulates the proliferation and differentiation of mesenchymal cells
46 Zhang et al. 1997. *Msx* genes play a critical role in craniofacial development, and *Msx1*^{−/−}
47 mouse embryos develop with a complete secondary cleft palate, as well as tooth agenesis. The
48 MSX1 protein harbors a HD sequence (amino acids 167–228), which interacts with target
49 proteins, such as octamer-binding transcription factor 1 (OCT1), TATA-binding protein (TBP),
50 distal-less homeobox (DLX), and enhancer of zeste 2 (EZH2) Zhang et al. 1997; Rave-Harel
51 et al. 2005.

52 Nuclear proteins translocate into the nucleus through nuclear pore complexes (NPCs)

53 Grunwald and Singer 2012. Small proteins can translocate through NPCs by passive diffusion,
 54 whereas active translocation is essential for larger proteins Lange et al. 2007. However, even
 55 small proteins, such as histones, promote translocation by an active process Breeuwer and
 56 Goldfarb 1990. These active processes are mediated by a nuclear localization signal (NLS)
 57 composed of basic amino acid residues Gorlich and Mattaj 1996. Classical monopartite or
 58 bipartite NLS sequences are recognized by the importin α and β heterodimer complex, and the
 59 proteins containing these moieties are translocated into the nucleus via an energy-dependent
 60 process Gorlich and Mattaj 1996; Lee et al. 2006. In classical NLS, importin α recognizes the
 61 NLS through a specific binding site and importin β mediates translocation of the protein
 62 through the NPCs. Furthermore, previous studies indicated that several homeoproteins, with
 63 unique NLS, required a different manner of interaction with importin α and/or β Ye et al. 2011.
 64 We hypothesize that MSX1 (33 kDa) may undergo nuclear localization in a similar manner to
 65 histones, and predict that functional NLS sites would be embedded in this protein.
 66 Several studies have suggested that the HD of MSX1 contains NLS sequences Wang et al.
 67 2011; Kimura et al. 2014. Although the HD is a highly conserved region, amino acid sequence
 68 divergence is observed in the NLS regions of homeoproteins Ye et al. 2011. In this study, we
 69 investigated the intracellular distribution of several MSX1 mutant proteins with deletions or

70 amino acid substitutions in cultured HEK 293 cells to identify the functional nuclear
71 localization motifs of MSX1. We identified two isolated basic motifs, located within the
72 N-terminus and C-terminus of the HD, and reveal that both the motifs are crucial for nuclear
73 localization of MSX1.

74

75 **Material and methods**

76 **Plasmid construction**

77 The cDNA encoding the MSX1 protein was amplified from human genomic DNA by PCR as
78 previously described Kamamoto et al. 2011. This cDNA fragment was then cloned into the
79 pcDNA3 vector (Invitrogen, Carlsbad, CA), harboring the FLAG epitope-tag at the 5' end of
80 the coding region. Site-directed mutagenesis and deletions were carried out using the PCR
81 megaprimer method and/or inverse PCR method, respectively (Supplemental table 1)
82 Kamamoto et al. 2011; Ochman et al. 1988. For detection of HD deletion mutants, we used
83 EGFP as the FLAG fusion proteins were not detected in the transfected cells, likely due to
84 protein instability (data not shown). To obtain an EGFP fusion protein, the fragments were
85 digested using *Bgl*II and *Sal*I and ligated into the pEGFP-C1 vector (Clontech, Mountain
86 View, CA). All constructs were verified by DNA sequencing.

87

88 **Immunolocalization of various MSX1 mutants**

89 Human embryonic kidney 293 (HEK 293; ATCC #CRL-1573) cells were cultured in

90 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine

91 serum at 37°C in an atmosphere of 5% CO₂. For transfection and immunostaining, cells were

92 plated onto 12-well poly-L-lysine-coated cover glass (12 mm in diameter; Matsunami, Osaka,

93 Japan) at a density of 10⁴ cells/well. After 2–3 days, transient transfections were carried out

94 using Lipofectamine 2000 (Invitrogen). 24 h after transfection, the cells were fixed with

95 3.75% (w/v) paraformaldehyde/phosphate buffered saline (PBS) and permeabilized with 0.2%

96 (v/v) Triton X-100/PBS and then incubated with the mouse anti-FLAG monoclonal antibody

97 (1:1000 dilution; Sigma-Aldrich, St. Louis, MO) or rabbit anti-GFP antibody (1:1000

98 dilution; Invitrogen), overnight at 4°C. The cells were subsequently incubated for 60 min at

99 room temperature with either Cy3-conjugated goat anti-mouse antibody (1:1000 dilution;

100 Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa Fluor[®] 488 donkey

101 anti-rabbit antibody (1:1000 dilution; Invitrogen). 4', 6-Diamidino-2-phenylindole (DAPI; 1

102 µg/mL) was used to detect nuclei.

103

104 **Microscopy and statistical analysis**

105 After washing three times with PBS, cells were visualized under an Olympus BH-2
106 microscope (Olympus, Tokyo, Japan). The images were captured using Photoshop Elements
107 11 software (Adobe, San Jose, CA) and analyzed using ImageJ Schneider et al. 2012 to
108 calculate the quantifying cellular distribution of MSX1 mutant proteins as previously
109 described, with minor modifications Gueroussov et al. 2010. Briefly, cells were analyzed by
110 measuring the average fluorescence of the fractions, which is manually separated nuclear,
111 cytoplasmic, and intracellular distribution, using the following equation:

$$\% \text{ of Nuclear fluorescence} = \left(\frac{A_{Nuc} \times (F_{Nuc} - F_{Back})}{A_{Tol} \times (F_{Tol} - F_{Back})} \right) \times 100$$

112 A_{Nuc} ; Area of the nucleus

113 A_{Tol} ; Area of the whole cell

114 F_{Nuc} ; Average fluorescence of the nuclear fraction

115 F_{Tol} ; Average fluorescence of the whole cell fraction

116 F_{Back} ; Average fluorescence of an untransfected cell

117 The percentage of nuclear fluorescence represents the mean $\pm$ standard error of the mean
118 (SEM). At least 50 cells were counted in five randomly selected fields in two or three
119 independent experiments. Statistical analysis was performed using the Welch's *t*-test. A

P-value of < 0.001 was considered a statistically significant difference between experimental groups.

Preparation of Nuclear and Cytosolic Extracts

Nuclear and cytosolic extracts were separated as previously described, with minor modifications Garcia-Yague et al. 2013. Briefly, the cell pellet was resuspended in three pellet volumes of cold buffer A (20 mM HEPES, pH 7.0, 0.15 mM EDTA, 0.015 mM EGTA, 10 mM KCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 µg/mL leupeptin) and incubated on ice for 30 min. Then the homogenate was centrifuged at $500 \times g$ for 5 min. The supernatants were taken as the cytosolic fraction. The nuclear pellet was resuspended in five volumes of cold buffer B (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 mM NaCl, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1 µg/mL leupeptin). After centrifugation, in the same conditions outlined above, the nuclei were resuspended in loading buffer containing 0.5% SDS.

137 **Western blotting**

138 The cytosolic and nuclear fractions were resolved by western blotting as previously described,
139 with minor modifications Tokita et al. 2001. Briefly, HEK 293 transfectants were extracted
140 with lysis buffer (1% Triton X-100, 1 mM EDTA in Tris-buffered saline; TBS) and these
141 lysates were run on an SDS-PAGE (12% gel) and transferred to Immobilon-P membranes
142 (Millipore Corporation, Bedford, MA). The membranes were blocked with 1% bovine serum
143 albumin (BSA) in TBS for 1 h at room temperature and then probed with an anti-GFP
144 antibody (1:2000, Invitrogen). Control experiments were performed using rabbit anti-histone
145 H3 K27me3 antibodies (1:3000, Millipore), and rabbit anti-alpha tubulin antibodies (1:1000,
146 Genetex, Irvine, CA). The signals on the blots were detected with horseradish
147 peroxidase-conjugated secondary antibodies (1:400, Vector Laboratories, Burlingame, CA)
148 and Western Lightning ECL Pro (PerkinElmer, Winter Street Waltham, MA).

149

150 **Results**

151 **Prediction of MSX1 nuclear localization signal**

152 The molecular weight of MSX1 protein is ~31 kDa, which means it can enter the nucleus by
153 passive diffusion. However, several reports have suggested that the nuclear localization of

MSX1 is dependent on its HD, since wild-type MSX1 and its entire HD was exclusively localized in the nucleus, and HD-deleted MSX1 (Δ HD) was only diffusely localized in the cytoplasm (Fig. 1) Wang et al. 2011; Kimura et al. 2014. These findings indicate that the HD is indispensable to nuclear localization and that one or more functional NLS sites are present in the MSX1 HD. NLS motifs were predicted using a web-based tool, NLStradamus Nguyen et al. 2009. We identified two putative NLS regions containing multiple positively charged residues at amino acids 161–170 and 216–223 within the HD. We designated them NLS1 ($_{161}$ RKHKTNRKPR $_{170}$) and NLS2 ($_{216}$ NRRAKAKR $_{223}$) (Fig. 1A).

Mapping of NLS sites in MSX1

We first constructed several EGFP-fused proteins of the MSX1 HD, both wild-type and deletion mutants. Whereas a significant reduction in the nuclear fluorescence signal intensity was observed for the FLAG- Δ HD when compared with FLAG-Wild type (Fig. 1C), FLAG-HD and EGFP-HD had similar nuclear fluorescence intensity to FLAG-Wild MSX1 (Fig. 1, 2). These results indicate that the nuclear localization of FLAG-HD, EGFP-HD, and FLAG-Wild MSX1 are partially directed by active transport, because the nuclear fluorescence intensity of these chimeric proteins is significantly higher than that of the EGFP, which can

171 enter the nucleus by passive diffusion Pan et al. 2004; Sato et al. 2013; Hatayama et al. 2008.
172 We then constructed EGFP fusion proteins with HDN'_{161–205} and HDC'_{205–228} (Fig. 2A). While
173 the immunolocalization of the NLS1-containing fusion protein (EGFP-HDN') was detected
174 predominantly in the nucleus, the NLS2-containing chimeric protein (EGFP-HDC') was
175 detected in the cytoplasm (Fig. 2B, C). Next, to determine whether the NLS1 or NLS2 were
176 required for nuclear localization of MSX1, we examined the subcellular distribution of the
177 EGFP fusion products of the HD mutants without NLS1 or NLS2, respectively (Fig. 2A). As
178 shown in Fig. 2B and C, the subcellular localization of EGFP-ΔNLS1 was similar to that of
179 EGFP, whereas EGFP-ΔNLS2 mutant HD was observed predominantly in the nucleus. As
180 shown in Fig. 2C, there are no significant differences between EGFP-ΔNLS1, EGFP-HDC',
181 and EGFP.

182

183 **Cooperative effect of the isolated NLS1 and NLS2 motifs**

184 To determine whether NLS1 alone is enough to direct the EGFP to the nuclei independently,
185 we analyzed the immunolocalization of the EGFP-NLS1, and two tandem NLS
186 (EGFP-NLS1+2; Fig. 3A). EGFP-NLS1+2 chimeric protein was predominantly localized in
187 the nucleus, whereas EGFP-NLS1 was observed in the cytoplasm, indicating that NLS2 is one

188 of the crucial signals for nuclear localization mediated by NLS1 (Fig. 3B, C). Interestingly,
189 there was equal nuclear fluorescence between EGFP-NLS1+2, EGFP-ΔNLS2, and
190 EGFP-HDN'. However, the nuclear fluorescence was significantly different between
191 EGFP-NLS1+2 and EGFP-HD (Fig. 3C). To validate the NLS potency of these two tandem
192 peptides biochemically, we performed western blotting analysis. While almost all EGFP
193 protein is detected in the cytosolic fraction, significant amounts of EGFP-NLS1+2 and
194 EGFP-HD proteins are detected in the nuclear fraction (Fig. 3D). These results demonstrated
195 that the two tandem NLS peptides promote nuclear localization.

196 Finally, to characterize the two tandem NLS peptides precisely, we performed site-directed
197 mutagenesis in NLS1+2 to generate mutants: EGFP-mNLS1N', EGFP-mNLS1C',
198 EGFP-mNLS1, EGFP-mNLS2, and EGFP-mNLS1+2, which have mutated the basic amino
199 acid residues to an Ala residue (Fig. 4A). Whereas the immunolocalization of
200 EGFP-mNLS1C' was observed mostly in the nucleus, the immunolocalization of both
201 EGFP-mNLS1N', EGFP-mNLS1, EGFP-mNLS2, and EGFP-mNLS1+2 was observed mostly
202 in the cytoplasm (Fig. 4B, C). There was no significant difference between EGFP-mNLS1N',
203 EGFP-mNLS1, EGFP-mNLS1+2, and EGFP. Interestingly, considerable nuclear localization
204 of EGFP-ΔNLS2 was observed (Fig. 2C), whereas only a small amount of nuclear

205 localization of EGFP-mNLS2 was observed (Fig. 4C).

206

207 **Discussion**

208 Generally, the folding structures of HDs are highly conserved and consist of three alpha

209 helices: helix-I, -II, and, -III. Helix-II and -III form a helix–turn–helix motif, and helix-III

210 recognizes specific DNA motifs Hovde et al. 2001; Damante et al. 1994. NLS sites in the HD

211 can be classified according to previous studies and our similar observations (Table 1).

212 Monopartite NLS sites within the helix-I or helix-III have been identified in some

213 homeoproteins such as Tst-1/Oct6, PDX-1, and PITX2, which are very similar to the NLS

214 sequence of SV40 T-antigen Sock et al. 1996; Kozlowski and Walter 2000; Moede et al. 1999.

215 Bipartite NLSs have a spacer between two interdependent basic amino acid residue clusters in

216 the N-terminal region of the HD, in close proximity to helix I. It has been reported that the

217 spacer peptide sequence of DLX3 is not necessary for nuclear localization Robbins et al.

218 1991; Bryan and Morasso 2000. Two NLSs are observed in Nanog, Nkx2.2 and TTF-1, and

219 are characterized by two independent NLS motifs in helix-I and helix-III, which can

220 cooperate for complete nuclear localization Do et al. 2007; Hessabi et al. 2000;

221 Christophe-Hobertus et al. 1999. However, these studies do not address the functions of the

222 spacer sequence between the two NLS motifs.

223 In our study, we reveal that MSX1 contains a characteristic tandem NLS, which are two basic

224 motifs located at the N-terminus of the HD and in helix-III. Although each of the predicted

225 NLSs, NLS1 (₁₆₁RKHKTNRKPR₁₇₀) and NLS2 (₂₁₆NRRAKAKR₂₂₃), is insufficient for

226 nuclear localization alone, when they cooperate there is considerable nuclear localization. We

227 demonstrate for the first time that a tandem arrangement of two isolated NLS motifs within a

228 HD can direct a foreign protein to the nucleus. Furthermore, we conclude that the peptide

229 sequence containing three basic amino acid residues in the N-terminus of NLS1 (₁₆₁RKHK₁₆₄)

230 is pivotal for the nuclear localization of human MSX1 since EGFP-ΔNLS1 and

231 EGFP-mNLS1N' were mostly detected in the cytoplasm and EGFP-mNLS1C' in the nucleus.

232 However, the GFP chimera with NLS1 alone has only partial potency. These results clearly

233 suggest that the N-terminus of NLS1 is insufficient alone, but plays a necessary part in the

234 NLS.

235 NLS2 cannot function by itself; nevertheless, it plays an important role in the NLS mediated

236 by the N-terminal of NLS1. This is because NLS2 greatly recovers nuclear localization, and

237 the alanine substitutions of NLS2 seriously affect the nuclear localization of the tandem NLS

238 chimera. EGFP-ΔNLS2 immunolocalization was detected mostly in the nucleus and EGFP

239 NLS1+2 fusion protein cannot fully recover the nuclear localization potency of the native
240 NLS. These observations imply that the spacer peptide sequence of NLS1+2 is necessary for
241 nuclear localization. The spacer sequence, for instance $_{183}\text{RKFRQK}_{188}$, close to NLS1, may be
242 important for nuclear localization as EGFP-HD'N showed predominant nuclear localization
243 compared with EGFP-NLS1.

244 It has been demonstrated that proteins with two NLSs require a strict tertiary structure to
245 function Ye et al. 2011; Wong et al. 2014. Thus, we predict that some alteration in the NLS
246 structure of the NLS1+2 chimeric GFP, because some proteins with two NLSs, such as
247 Nkx2.2, bind importin β directly after folding, whereas monopartite and bipartite NLSs can be
248 directly recognized either by importin α or importin β Ye et al. 2011. In the two NLSs of
249 TTF-1, the unique tripeptide Trp-Phe-Gln (WFQ), near the basic amino acid cluster in the
250 C-terminus of HD, is highly conserved across species. An alanine replacement in this
251 tripeptide affects the nuclear localization of the GFP chimeric protein Christophe-Hobertus et
252 al. 1999. Furthermore, a nonstop mutation prevents the nuclear localization of MSX1 with
253 intact HD due to an additional 48 amino acid residue at the C-terminus, which would likely
254 alter the protein's structure Wong et al. 2014. Based on the findings of these reports and our
255 study, we speculate that the three-dimensional structure is also crucial for full potency for the

two NLSs in human MSX1, and that the N-terminal of HD certainly would include other crucial peptide sequences which can improve the nuclear localization potency of NLS1. Finally, we demonstrate that two isolated basic motifs within the HD of MSX1 play pivotal roles in nuclear localization. Furthermore, our observations suggest that the NLS sequence can significantly promote nuclear localization of small proteins, such as MSX1. Though it has been previously demonstrated that the nuclear transports of several homeoproteins are mediated by importin families Ye et al. 2011, the molecular mechanism of nuclear transport of MSX1 was not known. Further studies are required to elucidate the precise molecular mechanisms of nuclear transport mediated by the two NLS sites in MSX1. Our study demonstrates a unique NLS motif in MSX1, which consists of an essential single core motif at the region close to helix-I with weak potency, and an auxiliary subdomain in the helix-III that alone does not contribute to nuclear localization potency.

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380 **Table. 1. NLS varies with the individual by the classification and location.**

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Figure captions

Figure 1. The nuclear localization of MSX1 depends on its HD. (A) Schematic diagrams of FLAG tagged wild-type MSX1, its deletion mutants, Δ HD and HD. The amino acid sequences of the putative NLS predicted by a web-based tool, NLStradamus (<http://www.moseslab.csb.utoronto.ca/NLStradamus/>) are shown. (B) The immunolocalization of FLAG tagged wild-type MSX1 and its deletion mutants in HEK293 cells were detected using the mouse anti-FLAG antibody and Cy3-conjugated anti-mouse IgG (Red). Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20 μ m. (C) Photographs of randomly selected fields were taken under the same conditions, and all cells within each field were scored according to signal intensity distribution in the nucleus and cytoplasm (see materials and methods). The percentage of nuclear fluorescence represents the mean $\pm$ SEM from two experiments in which 50 cells were scored per experiment. The p-values compare the percentage of nuclear fluorescence in the control versus each HD protein mutants according to the Welch's t test. *** $p > 0.001$ versus each fluorescence.

Figure 2. Mapping of the NLS motifs in the MSX1 protein. (A) Schematic diagram of EGFP tagged MSX1-HD and its deletion mutants. Deletion mutants of the EGFP MSX-HD

399 fusion protein (HDN'₁₆₁₋₁₉₁, HDC'₁₉₂₋₂₂₈, ΔNLS1 and ΔNLS2) were constructed. **(B)**
400 Subcellular localization of these mutant fusion proteins detected using anti-GFP antibody and
401 Alexa488-labeled secondary antibodies (Green). Nuclear DNA was counterstained with DAPI
402 (Blue). Scale bar; 20μm. **(C)** The percentage of nuclear fluorescence of the chimeric proteins.
403 The values represent the mean ± SEM from two experiments in which 50 cells were scored
404 per experiment. The p-values compare the percentage of nuclear fluorescence in the control
405 versus each HD protein mutants according to the Welch's t test. *** $p > 0.001$ versus
406 EGFP-HD and # $p > 0.001$ versus EGFP.

407
408 **Figure 3. Cooperative effect of the isolated NLS1 and 2 motifs.** **(A)** Schematic diagram of
409 EGFP fusion proteins of an entire HD. NLS1 and NLS1+2 were constructed. **(B)** Subcellular
410 localization of mutant fusion proteins. The subcellular localization of EGFP-fusion proteins
411 was detected using anti-GFP antibody and Alexa488-labeled secondary antibodies (Green).
412 Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20μm. **(C)** The percentage of
413 nuclear fluorescence of the chimeric proteins. The values represent the mean ± SEM from two
414 experiments in which 50 cells were scored per experiment. The p-values compare the
415 percentage of nuclear fluorescence in the control versus each HD protein mutants according

to the Welch's t test. *** $p > 0.001$ versus EGFP-HD and # $p > 0.001$ versus EGFP. **(D)**

Western blot analysis for EGFP was performed on cytosolic and nuclear extracts prepared

from HEK293 cells transfected transiently with EGFP EGFP-HD or EGFP-NLS1+2 (Upper

panel). Histone H3 and tubulin were used as loading controls (lower panels). Nuclear:

Nuclear fraction, Cytosolic: Cytosolic fraction.

Figure 4. Characterization of the tandem two NLS motifs in MSX1. (A) Schematic

diagram of the mutants of the EGFP NLS1+2 fusion protein (mNLS1N', mNLS1C', mNLS1,

mNLS2 and mNLS1+2) were constructed. **(B)** Subcellular localization of mutant fusion

proteins. The subcellular localization of EGFP-fusion proteins was detected using anti-GFP

antibody and Alexa488-labeled secondary antibodies (Green). Nuclear DNA was

counterstained with DAPI (Blue). Scale bar; 20 μ m. **(C)** The percentage of nuclear

fluorescence of the chimeric proteins. The values represent the mean $\pm$ SEM from two

experiments in which 50 cells were scored per experiment. The p-values compare the

percentage of nuclear fluorescence in the control versus each HD protein mutants according

to the Welch's t test. *** $p > 0.001$ versus EGFP-NLS1+2 and # $p > 0.001$ versus EGFP.

433 **Supporting Information**

434 S1 Table. Oligonucleotide primers for mutagenesis of MSX1 HD

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Figure 1. The nuclear localization of MSX1 depends on its HD. (A) Schematic diagrams of FLAG tagged wild-type MSX1, its deletion mutants, ΔHD and HD. The amino acid sequences of the putative NLS predicted by a web-based tool, NLStradamus (<http://www.moseslab.csb.utoronto.ca/NLStradamus/>) are shown. (B) The immunolocalization of FLAG tagged wild-type MSX1 and its deletion mutants in HEK293 cells were detected using the mouse anti-FLAG antibody and Cy3-conjugated anti-mouse IgG (Red). Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20μm. (C) Photographs of randomly selected fields were taken under the same conditions, and all cells within each field were scored according to signal intensity distribution in the nucleus and cytoplasm (see materials and methods). The percentage of nuclear fluorescence represents the mean ± SEM from two experiments in which 50 cells were scored per experiment. The p-values compare the percentage of nuclear fluorescence in the control versus each HD protein mutants according to the Welch's t test. *** p > 0.001 versus each fluorescence.

180x154mm (300 x 300 DPI)

Figure 2. Mapping of the NLS motifs in the MSX1 protein. (A) Schematic diagram of EGFP tagged MSX1-HD and its deletion mutants. Deletion mutants of the EGFP MSX-HD fusion protein (HDN'161-191, HDC'192-228, ΔNLS1 and ΔNLS2) were constructed. (B) Subcellular localization of these mutant fusion proteins detected using anti-GFP antibody and Alexa488-labeled secondary antibodies (Green). Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20μm. (C) The percentage of nuclear fluorescence of the chimeric proteins. The values represent the mean ± SEM from two experiments in which 50 cells were scored per experiment. The p-values compare the percentage of nuclear fluorescence in the control versus each HD protein mutants according to the Welch's t test. *** p > 0.001 versus EGFP-HD and # p > 0.001 versus EGFP.

167x139mm (300 x 300 DPI)

Figure 3. Cooperative effect of the isolated NLS1 and 2 motifs. (A) Schematic diagram of EGFP fusion proteins of an entire HD. NLS1 and NLS1+2 were constructed. (B) Subcellular localization of mutant fusion proteins. The subcellular localization of EGFP-fusion proteins was detected using anti-GFP antibody and Alexa488-labeled secondary antibodies (Green). Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20μm. (C) The percentage of nuclear fluorescence of the chimeric proteins. The values represent the mean $\pm$ SEM from two experiments in which 50 cells were scored per experiment. The p-values compare the percentage of nuclear fluorescence in the control versus each HD protein mutants according to the Welch's t test. *** $p > 0.001$ versus EGFP-HD and # $p > 0.001$ versus EGFP. (D) Western blot analysis for EGFP was performed on cytosolic and nuclear extracts prepared from HEK293 cells transfected transiently with EGFP EGFP-HD or EGFP-NLS1+2 (Upper panel). Histone H3 and tubulin were used as loading controls (lower panels). Nuclear: Nuclear fraction, Cytosolic: Cytosolic fraction.

195x130mm (300 x 300 DPI)

Figure 4. Characterization of the tandem two NLS motifs in MSX1. (A) Schematic diagram of the mutants of the EGFP NLS1+2 fusion protein (mNLS1N', mNLS1C', mNLS1, mNLS2 and mNLS1+2) were constructed. (B) Subcellular localization of mutant fusion proteins. The subcellular localization of EGFP-fusion proteins was detected using anti-GFP antibody and Alexa488-labeled secondary antibodies (Green). Nuclear DNA was counterstained with DAPI (Blue). Scale bar; 20µm. (C) The percentage of nuclear fluorescence of the chimeric proteins. The values represent the mean ± SEM from two experiments in which 50 cells were scored per experiment. The p-values compare the percentage of nuclear fluorescence in the control versus each HD protein mutants according to the Welch's t test. *** p > 0.001 versus EGFP-NLS1+2 and # p > 0.001 versus EGFP.

147x106mm (300 x 300 DPI)

Table. 1. NLS varies with the individual by the classification and location.

Protein	Classification	Location of NLS		Reference
		N-terminus of HD	C-terminus of HD	
Tst-1/Oct6	monopartite	GRKRKKRT		Sock et al. 1996
PITX2	monopartite		RVRVWFKNRRRAKWRKR	Kozlowski and Walter 2000
PDX1	monopartite		RRMKWKK	Moede et al. 1999
Dlx3	bipartite	GKPKKVRK..14A.a..RRFK		Bryan and Morasso 2000
Nanog	two NLS	KQKTR	RMKSKRWQ	Do et al. 2007
Nkx2.2	two NLS	KKRKRR	RYKMKRAR	Hessabi et al. 2000
TTF-1	two NLS	PRRKRRV	WFQ	Christophe-Hobertus et al. 1999
MSX1	two NLS	RKHKTNRKPR	RRAKAKR	This study

HD: Homeodomain, NLS: Nuclear localization signal

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S1 Table. Oligonucleotide primers for mutagenesis of MSX1 HD

Name/ template	Primer		PCR methods
	5' terminus	3' terminus	
pcDNA3			
entire HD	AGATCTCGCAAACACAAGACGAACCG	GAGACTACAAGAGGCAGAGTAGGTCCAG	Conventional
HDN'	CAGGTACTGCTTCTGGCGGAAC	TAGCTCGAGCATGCATCTAGAGG	Inverse
HDC'	ATGGACTACAAGGACGACGACAAG	TCCATCGCCGAGCGCGGAGTT	Inverse
EGFPC1			
HDN'	AGATCTCGCAAACACAAGACGAACCG	CGCCAGAAGCAGTACCTGTAGGTCCAG	Conventional
HDC'	AGATCTTCCATCGCCGAGCGCGC	GAGACTACAAGAGGCAGAGTAGGTCCAG	Conventional
ΔNLS1	CGAGCTGTACAAGTCCGGACTCAGATCT	ACGCCCTTACCACCGCGCAGCTG	Inverse
ΔNLS2	ACGCAGGTGAAGATATGGTTCCGAAC	CAAGAGGCAGAGTAGGTCCAGATCACT	Inverse
NLS1+2	CAAAACAAGACGAACCGTAAGCCGCGG	CGCCGCGCCAAGGCAAAGAGACTACAA	Inverse
mNLS1N'	1st GATCCAGATCTGCCGC ACACAAGACGA		Mega
	2nd AGATCTGCCGCACACGCGACGA ACCGT		Mega
mNLS1C'	1st CCGTAAGCCGGCGAACCCGCGGCCA		Mega
	2nd ACAAGACGAACGCTGCCGCGCGAAC		Mega
mNLS2	1st CGCCAAGGACGCGCACTACAAGAGG		Mega
	2nd AACCGCCGCGCCGCGGACGCGCACT		Mega
	3rd TAAGCCGCGGAACGCCGCGCGCGGCA		Mega
NLS1	ACGCGACGAACGCTGCCGCGCGAAC		Mega
NLS1+2	TGCGCCGCGGAACGCCGCGCGCGGCA		Mega
mega primer 5'terminus	ATTGATAAGTACTTGAAATCCAGCAAGT		-
mega primer 3'terminus	TCAGTTATCTAGATCCGGTGGATCCCG		-

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