THE EMBO JOURNAL

Manuscript EMBO-2010-73945

An actin-regulated importin α/β-dependent extended bipartite NLS directs nuclear import of MRTF-A

Rafał Pawłowski, Eeva Kaisa Rajakylä, Maria K. Vartiainen, and Richard Treisman

Corresponding author: Richard Treisman, Cancer Research UK London Research Institute

Review timeline:

Submission date:	15 February 2010
Editorial Decision:	25 March 2010
Revision received:	26 July 2010
Editorial Decision:	06 August 2010
Accepted:	06 August 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 March 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise again for the time taken to get back to you with a decision - as I told you, this was due to a long delay in receiving the final report on your manuscript. However, we do now have the comments from all three referees, which are enclosed below. As you will see, all three referees are broadly positive, but while referee 2 does not provide a real report and requests no changes, both referees 1 and 3 raise significant concerns that will need to be addressed by a major revision of the manuscript. Most notably, both referees find that further evidence for the physiological relevance of the reported regulation of MRTF-A nuclear import would be essential for eventual publication in the EMBO Journal. In this sense, it will be particularly important that you conclusively address the major point of referee 1, as well as point 2 of referee 3.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. Since you told us that there is a competing study, it would be very helpful if you contact us if this manuscript (or any other related paper) is published, so that we can discuss how best to proceed rapidly with your manuscript. I hope that you should be

able to complete the necessary revisions well within the three-month period, but again, please let us know if you foresee any problems with meeting this deadline.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the manuscript entitled "An actin-regulated importin α/β-dependent extended bipartite NLS directs nuclear import of MRTF-A", Pawlowski et al. show that MRTF2-A has a regulated, unusually long classical bipartite NLS. Using a combination of microscopy studies and biochemical pull-downs, they demonstrate that each of the basic regions is necessary for proper nuclear relocalization, that both basic regions are sufficient for nuclear import, that nuclear localization is dependent on the presence of importin , that importin directly interacts with MRTF-A in a manner that is dependent on the basic regions, and that MRTF-A/importin interaction can be disrupted with actin suggesting a method of regulation in vivo. This paper meets all of the criteria for establishing that a putative NLS is a true targeting signal. This study should be of significant interest to a broad range of readers due to the unusual nature of the MRTF-A bipartite cNLS linker region and the elucidation of a possible mechanism for the regulated relocalization of MRTF-A in response to actin polymerization.

The one major criticism is that the authors do not prove the link between the cNLS identified and the biological function of MRTF-A. Ideally, the authors would demonstrate the requirement for this cNLS sequence in the function of MRTF-A in cells by knocking down the endogenous protein and assessing the function of some of the variants characterized in this study. This experiment may be a major undertaking. Perhaps there are alternative functional assays that could be employed but some evidence that the cNLS is required for a biological function of MRTF-A is required.

The following minor edits to the manuscript are also suggested:

-page 5, paragraph 1: Please clarify which N-terminal sequences of MRTF-A have previously been shown to be necessary and sufficient for shuttling.

-page 5, paragraph 2: Should be "Figure 1B", not "Figure B2".

-page 6, paragraph 1: In Figure 1C, B3A MRTF-A accumulates in the nucleus after extended treatment with LMB. The authors interpret this result as suggesting that the alanine substitutions do not completely inactivate the B3 element. The other interpretation is that the B2 element can at least partially function as a monopartite cNLS. Later experiments show that the B2 element alone cannot mediate import of a normally cytoplasmic protein, but the possibility still exists as of this experiment and should be addressed here.

-page 6, paragraph 2: The authors show that mutations in each of three successive triplets in the B2 region can impair nuclear accumulation. Two of the triplets involve basic residues in the region and fit with current ideas about classical NLSs. However, altering the amino acids "LAD" also affected the nuclear import. Please discuss possible explanations for why this might occur.

Referee #2 (Remarks to the Author):

This manuscript provides important mechanistic insight into the regulation of nuclear import associated with the transcriptional co-factor MRTF-A. The identification of importin-dependent

nuclear translocation of this important transcriptional co-factor is of interest to a broad readership. Although importin-mediated control of nuclear transportation is not new, the present study identifies G-actin as a competitive regulator of importin action. This is novel and warrants publication in the EMBO Journal.

Referee #3 (Remarks to the Author):

In this manuscript, the authors attempted to know how myocardin-related transcription factor-A (MRTF-A) is transported into the nucleus and how its import is regulated by G-actin. First, they demonstrated that two separated basic amino acid clusters function as a bipartite type nuclear localization signal (NLS) of WRTF-A. In response to serum stimulation or LMB treatment, wild type MRTF-A translocated into the nucleus whereas mutant NLS-containing MRTF-A stayed in the cytoplasm. Furthermore, they showed that the classical nuclear import machinery consisting of importin alpha/beta was involved in the MRTF-A nuclear localization using in vitro transport assay and RNAi experiment. They also demonstrated that importin alpha directly bound to MRTF-A NLS which was located in its actin binding domain and that G-actin competed with importin alpha to bind to MRTF-A in vitro, which explains the actin-regulated nuclear translocation of MRTF-A.

The experiments are well planed and most of the data support the proposed conclusion. Several critical points, however, should to be addressed before publication in EMBO Journal.

Specific points:

1) Participation of importin alpha in MRTF-A nuclear import is not clear. Reconstituted in vitro nuclear transport assay using recombinant proteins (MRTF-A, importin alpha/beta, Ran-GDP, NTF2 and ATP regeneration system) should be performed to obtain the direct evidence showing that importin alpha is actually involved, and that importin alpha family members participate in the MRTF-A nuclear transport.

2) One of the most important findings in this work is that reduced G-actin caused free MRTF-A which can be recognized by importin alpha, resulting in the nuclear import of MRTF-A. To confirm this, the authors should show in vivo evidence that MRTF-A actually interacts with importin alpha/beta in cells in response to serum stimulation.

3) Although Crm1-mediated nuclear export is dominant in resting cells, MRTF-A shuttles between the nucleus and the cytoplasm. If the authors' conclusion is correct, G-actin should bind to MRTF-A and inhibit its nuclear import in the resting cells. On the other hand, importin beta-knockdown showed marked effects on serum-stimulated nuclear import of MRTF-A, but weaker effects on leptomycin B- or cytochalasin D-treated cells as shown in Fig. 3A, which means that constitutive and conditional nuclear import pathways for MRTF-A exist in cells. Could these results be addressed only by classical importin alpha/beta pathway?

Minor points:

1) The authors use "unusually extended NLS" to explain bipartite type NLS of MRTF-A. Although most of the bipartite type NLSs identified so far contain 10-12 residues spacer, NLSs containing a longer linker have been emerging as follows. The authors should mention this issue more appropriately.

Recent reports:
Traffic, 11, 311-323 (2010)
J. Cell Sci., 123, 23-28 (2010)
J. Virol., 83, 12842-12853 (2009)

2) Does 111-166 fragment of MRTF-A bind to G-actin and has the ability of serum-dependent nuclear import?

3) Fig. 5D: Can importin alpha compete with binding of actin to MRTF-A?

4) Fig. 1C and D: error bars should be shown as other graphs.

5) Page 5, line 17: "Figure B2" should be "Figure 1B".

6) Page 7, line 14: an unpaired parenthesis exists.

7) Page 11, line 8: "importin beta" should be "importin alpha".

8) Page 21, line 3: "importin R activity" should be "importin beta activity".

1st Revision - Authors' Response 26 July 2010

REVIEWER 1

We were pleased to see that the reviewer considered the paper to be of significant interest and thank him/er for the helpful comments and suggestions. Prompted by his comments we conducted further studies on the role of importin alpha/beta and nature of its interaction with the NLS, which we have included in the paper. We have revised the paper in the light of his comments and have included new data on functional analysis of the import signal and its interaction with importin alpha/beta in vitro and in vivo

MAJOR POINT

> *The one major criticism is that the authors do not prove the link between the cNLS identified and the biological function of MRTF-A. Ideally, the authors would demonstrate the requirement for this cNLS sequence in the function of MRTF-A in cells by knocking down the endogenous protein and assessing the function of some of the variants characterized in this study. This experiment may be a major undertaking. Perhaps there are alternative functional assays that could be employed but some evidence that the cNLS is required for a biological function of MRTF-A is required.*

We have used RNAi knockdown to deplete cells of both MRTF-A and MRTF-B, which substantially reduces both basal and induced activity of a cotransfected SRF reporter. Expression of RNAi-resistant MRTF-A rescues both activities, although not to the extent of wildtype cells; while basal activity is increased by MRTF-A re-expression, induced activity remains considerably less than in non-depleted cells (this may reflect "squelching" from overexpression of the strong myocardin-family activation domain under stimulation conditions - Miralles 2003 Cell 113, p329; Wang 2001 Cell 105, p851). To exclude the effects of the expressed MRTF NLS mutant accessing the nucleus through dimerization with endogenous residual MRTFs, we tested the NLS mutant in context of both wildtype MRTF-A and MRTF-A lacking its leucine zipper sequence. Rescue of both basal and induced activities is dependent on the NLS element within the RPEL domain, and the dependence is more marked in the zipper mutant. Data added as new Figure 1E, text on Results p6.

Sequences and factors required for its nuclear entry are thus obviously of biological significance for a transcriptional coactivator that is predominantly cytoplasmic but accumulates in the nucleus following signal, and our accumulation and import assays in vitro directly address this.

Minor points

> *-page 6, paragraph 1: In Figure 1C, B3A MRTF-A accumulates in the nucleus after extended treatment with LMB. The authors interpret this result as suggesting that the alanine substitutions do not completely inactivate the B3 element. The other interpretation is that the B2 element can at least partially function as a monopartite cNLS. Later experiments show that the B2 element alone cannot mediate import of a normally cytoplasmic protein, but the possibility still exists as of this experiment and should be addressed here.*

We have now performed a detailed analysis of this idea. We first tested the behaviour of the peptide-PK fusions. As the referee suggests, the peptide containing the B2 element alone indeed exhibits autonomous import activity, revealed upon treatment with LMB; in contrast the B3 element does not, either alone or in the context of the B2+B3 peptide with the B2 element mutated (revised

Figure 2, discussed on Results p7 §2). Similar results have been reported in the case of the BIV Rev protein, which also exhibits an extended bipartite NLS (Corredor and Archambault, 2009 JVirol 83, p12842), which is now cited in the Results (p7), and the implications are addressed in the Discussion (pp14-15).

Nevertheless, both basic elements are required for efficient import in the context of intact MRTF-A (Figure 1), and most importantly, high affinity binding of Impa to the extended NLS requires both its major and minor binding pockets (new Figure 5C; results p11). In the MRTF-A atypical extended NLS a weak interaction between Impa and the B2 element, probably through the major binding pocket is thus likely to be potentiated by additional interactions with the B3 basic sequences, probably mediated by the Impa minor binding pocket. These results, together with other recent findings, begin to blur the distinction between bipartite and monopartite NLS (revised Discussion p14-15).

-page 6, paragraph 2: The authors show that mutations in each of three successive triplets in the B2 region can impair nuclear accumulation. Two of the triplets involve basic residues in the region and fit with current ideas about classical NLSs. However, altering the amino acids "LAD" also affected the nuclear import. Please discuss possible explanations for why this might occur.

We used a peptide array, presented as Figure S2 in the submission, to show that acidic residues at these positions are required for effective interaction of recombinant Impa with MRTF-A NLS peptides. This may reflect interactions with Impa surfaces outside the previously characterised binding pockets; indeed in some contexts acidic NLS linker residues may favour interactions with Impa surface between the pockets (Kosugi et al. 2008, 2009, now cited Discussion page 15).

In experiments presented in the revised paper we used site-directed mutagenesis to confirm that the major and minor binding pockets of Impa are required for interaction with the MRTF-A extended NLS (Figure 5C, results page 11).

-page 5, paragraph 1: Please clarify which N-terminal sequences of MRTF-A have previously been shown to be necessary and sufficient for shuttling.

Reworded to clarify (page 5 §1 first sentence)

-page 5, paragraph 2: Should be "Figure 1B", not "Figure B2".

Done.

REVIEWER 2

We were gratified to find that the Reviewer found that "...identification of importin-dependent nuclear translocation of this important transcriptional co-factor is of interest to a broad readership", and that the study "... is novel and warrants publication in the EMBO Journal."

REVIEWER 3

We were pleased to see that the referee thought that "..the experiments are well planed and most of the data support the proposed conclusion."

Major points:

1) Participation of importin alpha in MRTF-A nuclear import is not clear. Reconstituted in vitro nuclear transport assay using) should be performed to obtain the direct evidence showing that importin alpha is actually involved, and that importin alpha family members participate in the MRTF-A nuclear transport.

We have now done this experiment using the permeabilised cells system with the retic lysate replaced with recombinant proteins (MRTF-A, Impa3, Impb, Ran.GDP RanBP1, RanGAP and NTF2). Import in vitro was observed only in the presence of both both Impa3 and Impb (New Figure 4C, text p10).

2) One of the most important findings in this work is that reduced G-actin caused free MRTF-A which can be recognized by importin alpha, resulting in the nuclear import of MRTF-A. To confirm this, the authors should show in vivo evidence that MRTF-A actually interacts with importin alpha/beta in cells in response to serum stimulation.

We showed previously that MRTF-A nuclear import occurs constitutively in fibroblasts and that its rate does not increase upon G-actin depletion, so stimulation would not be expected to increase interaction; however, import is inhibited upon overexpression of G-actin (Vartiainen et al, Science 316, p1749 (2007)). We were unable to detect MRTF-A-importin interactions using co-IP, but instead used a proximity ligation assay to detect interactions between endogenous Impb and MRTF-A. The assay, which uses localized fluorescent rolling-circle amplification (RCA) to detect short range interactions between antibodies in fixed cells, demonstrates that endogenous Impb and MRTF-A do associate in vivo, and that association is decreased in cells transfected with a b-actin expression vector (New Figure 3E, Results p9).

3) Although Crm1-mediated nuclear export is dominant in resting cells, MRTF-A shuttles between the nucleus and the cytoplasm. If the authors' conclusion is correct, G-actin should bind to MRTF-A and inhibit its nuclear import in the resting cells. On the other hand, importin beta-knockdown showed marked effects on serum-stimulated nuclear import of MRTF-A, but weaker effects on leptomycin B- or cytochalasin D-treated cells as shown in Fig. 3A, which means that constitutive and conditional nuclear import pathways for MRTF-A exist in cells. Could these results be addressed only by classical importin alpha/beta pathway?

The result in Figure 3A could indeed be interpreted that stimulated import is Impb dependent, while basal import is Impb-independent, and we now mention this (Results p8). However, two arguments lead us to the conclusion that the simplest explanation for our data is that the Impa-Impb dependent NLS mapped here is involved in both situations.

(1) The extended NLS scores both as the signal required for MRTF-A import in resting cells treated with LMB, and for nuclear accumulation in serum-stimulated cells, and our in vitro data show that Impa-Impb dimers are necessary and sufficient for its function.

(2) Our previous analysis of MRTF-A nuclear accumulation dynamics (Vartiainen et al, 2007) showed that (i) MRTF-A nuclear import occurs constitutively in fibroblasts, and its rate does not increase upon signal-induced G-actin depletion, showing that under resting conditions actin binding does not inhibit import; (ii) LMB or Cytochalasin D treatment both completely block export, while FCS stimulation reduces export but does not abolish it. Given these observations it would be expected that the apparent effect of Impb knockdown on net MRTF-A nuclear accumulation would be greater for stimuli that block export than those that merely reduce it, which is indeed the case.

The revised text explains these arguments more clearly (Results p8). The possibility that there are independent NLS involved in basal import and following stimulation mentioned in the revised Discussion §1 (p13).

Minor points:

1) The authors use "unusually extended NLS" to explain bipartite type NLS of MRTF-A. Although most of the bipartite type NLSs identified so far contain 10-12 residues spacer, NLSs containing a longer linker have been emerging as follows. The authors should mention this issue more appropriately.

We have expanded our discussion of this issue. It is now mentioned in the Introduction (p4 §1), in Results concerning autonomous NLS function of the two basic elements (p7 §2), and in the Discussion, from the points of view of NLS structure (p13 §1) and impa-NLS recognition function (p14-15). The additional references are cited.

2) Does 111-166 fragment of MRTF-A bind to G-actin and has the ability of serum-dependent nuclear import?

While peptides including RPEL2, such as the 111-166 fragment studied here, do bind actin in vitro (Kd ~2mM), the integrity of all three RPEL motifs is required for correct regulation of MRTF-A in response to serum stimulation (Guettler et al MCB 28 p732 (2008)). The apparent actin-binding affinity of the intact RPEL domain is ~50nM (Posern et al, EMBO, 23, p3973 (2004)). It is likely that this reflects cooperativity in assembly of complexes containing multiple actins on the entire domain; we have observed a complex containing three actins in gel filtration experiments (Vartiainen et al, Science 316, p1749 (2007)). Consistent with these observations, overexpression of actin cannot relocalise the 111-166-PK fusion protein to the cytoplasm (cited in Results p7).

3) Fig. 5D: Can importin alpha compete with binding of actin to MRTF-A?

Yes. Both the actin-Impa and Impa-actin competition experiments for MRTF-A are now shown separately in a new Figure 6 (text p12).

4) Fig. 1C and D: error bars should be shown as other graphs.

Done.

5) Page 5, line 17: "Figure B2" should be "Figure 1B".

6) Page 7, line 14: an unpaired parenthesis exists.

7) Page 11, line 8: "importin beta" should be "importin alpha".

8) Page 21, line 3: "importin R activity" should be "importin beta activity".

Corrected.

2nd Editorial Decision 06 August 2010

Many thanks for submitting the revised version of your manuscript to the EMBO Journal. It has now been seen again by referees 1 (who had no specific comments) and 3, whose comments are enclosed below. As you will see, both are happy with the revision, and I am therefore pleased to be able to tell you that we can accept your manuscript for publication, without the need for any further changes. You should receive the formal acceptance message shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee 3:

The authors have appropriately revised the manuscript according to my suggestions. Therefore, the revised version warrants publication in the EMBO Journal.

