Nuclear Import and Export Signals of Human Cohesins SA1/STAG1 and SA2/STAG2 Expressed in *Saccharomyces cerevisiae*

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Abstract

**Background:** Human SA/STAG proteins, homologues of the yeast Irr1/Scc3 cohesin, are the least studied constituents of the sister chromatid cohesion complex crucial for proper chromosome segregation. The two SA paralogues, SA1 and SA2, show some specificity towards the chromosome region they stabilize, and SA2, but not SA1, has been shown to participate in transcriptional regulation as well. The molecular basis of this functional divergence is unknown.

**Methodology/Principal Findings:** In *silico* analysis indicates numerous putative nuclear localization (NLS) and export (NES) signals in the SA proteins, suggesting the possibility of their nucleocytoplasmic shuttling. We studied the functionality of those putative signals by expressing fluorescently tagged SA1 and SA2 in the yeast *Saccharomyces cerevisiae*. Only the N-terminal NLS turned out to be functional in SA1. In contrast, the SA2 protein has at least two functional NLS and also two functional NES. Depending on the balance between these opposing signals, SA2 resides in the nucleus or is distributed throughout the cell. Validation of the above conclusions in HeLa cells confirmed that the same N-terminal NLS of SA1 is functional in those cells. In contrast, in SA2 the principal NLS functioning in HeLa cells is different from that identified in yeast and is localized to the C-terminus.

**Conclusions/Significance:** This is the first demonstration of the possibility of non-nuclear localization of an SA protein. The reported difference in the organization between the two SA homologues may also be relevant to their partially divergent functions. The mechanisms determining subcellular localization of cohesins are only partially conserved between yeast and human cells.

Introduction

Division of the eukaryotic cell requires exact distribution of a proper number of chromosomes into both daughter cells during mitosis. This involves, among others, tight coupling of sister chromatids until the early or mid-phase of mitosis and then their concerted separation at the onset of anaphase. One of the principal mechanisms responsible for the association of chromatids prior to their segregation is sister chromatid cohesion that relies on a complex of proteins highly conserved from yeasts to mammals, called cohesin. This complex consists of four core subunits: the structural maintenance of chromosomes (SMC) proteins SMC1 (Sml1 in the yeast *Saccharomyces cerevisiae*) and SMC3 (Sme3), the kleisin SCC1/RAD21 (Scc1/Rad21/Mcd1), and the least studied HEAT-repeat domain subunit called Irr1/Scc3 in yeast and SA/STAG or stromalins in humans. The basic function of the SA proteins has been elucidated owing to findings on the role of their yeast orthologue Irr1p [1]. Human cells contain two mitotic equivalents of Irr1, SA1 and SA2 (also called STAG1 and STAG2), which are present in two distinct 14S cohesin complexes [2–4]. Initially it was shown that these two complexes differ by their SA constituent, SA1 or SA2, and it was assumed that only one type of SA is present in a given cell [2], [3], [6]. However, subsequent data obtained by Canudas and Smith [7] indicated that both SA1 and SA2 were in fact present in one cell and were specifically required for the cohesion of chromosome arms and telomeres, and centromeres, respectively. Recent stoichiometry data indicate that in HeLa cells the ratio of complexes containing SA1 and SA2 is in the range between 1:12 and 1:15 [8].

Several topological models of sister chromatid cohesion have been proposed [9]. The most popular one-ring model assumes that the complex surrounds the replicated sister chromatids, with SMC1 and SMC3 creating a V-shaped heterodimer bridged by SCC1 [10]; [11]. Another model posits that the complex consists of two cohesin rings, each encircling...
a single chromatid, that are paired through an interaction of the C-terminal domain of Scc1 with SA [6].

In mammalian cells most cohesin complexes are associated with chromatin. However, a large fraction of cohesin on chromosome arms dissociates already during prophase, while the cohesin at the centromeres remains bound until the metaphase-to-anaphase transition. Gerlich et al. [12] identified three sub-populations of cohesin complexes whose SA proteins differed in their dynamic equilibrium rate between chromatin-bound and soluble state. Also in yeast the core cohesin subunits are dynamic. They are able to bind to and dissociate from chromatin and, potentially, to dissociate/associate from the whole cohesin complex in vivo. These events take place on a time scale less than a cell cycle, but without the loss of chromosome cohesion [13]. Metazoan cells undergo open mitosis with the microtubule-organizing centers, centrosomes, located outside the nucleus. At the onset of mitosis (prometaphase), the nuclear envelope breaks down and nuclear pore complexes disassemble. This enables microtubules of the spindle to interact with centrosomes [14], [15]. Also at this stage of mitosis a large fraction of cohesin dissociates from chromosome arms.

In addition to its originally identified function in ensuring cohesion of chromatids, the SA2 protein takes part in regulating transcription, where it can function as a co-activator [16] as well as element of the insulator complex [17]. The molecular mechanisms enabling such diverse activities and the functional distinction between SA1 and SA2 are poorly understood.

We undertook to address these questions by using a simplified model of the yeast cell for initial experiments. Budding yeast, as many other single-cell Eukaryota, undergo closed mitosis throughout their nuclear envelope remains intact [18]. Although closed mitosis is different from the open one in a number of features, the fundamental mechanisms of chromosome segregation are preserved, and the continuous presence of the nuclear envelope facilitates studies of a crucial aspect of the process, namely nuclear-cytoplasmic distribution of proteins. We took advantage of this aspect of yeast mitosis and also of the fact that it expresses only a single SA orthologue to study functional differences between the human SA paralogues. SA1 and SA2 were expressed individually in S. cerevisiae and their subcellular localization was investigated. Since our in silico analysis indicated that several nuclear localization signals (NLS) are present in SA1 and SA2, we investigated their functionality. Unexpectedly, also putative nuclear export signals (NES) were detected, suggesting the possibility that the SA proteins could shuttle between the nucleus and the cytoplasm. We showed that the SA2 protein, but not SA1, can indeed be exported from the nucleus using a Crm1-dependent route. Experiments in HeLa cells were performed to validate the yeast data. They confirmed the functionality of the NLS signal identified in SA1, but the import and putative export of SA2 turned out to be more complex than in yeast.

Since the Crm1-dependent export pathway is conserved in humans we propose that nuclear export can also be used to dissociate/associate from the whole cohesin complex in vivo. These events take place on a time scale less than a cell cycle, but without the loss of chromosome cohesion [13]. Metazoan cells undergo open mitosis with the microtubule-organizing centers, centrosomes, located outside the nucleus. At the onset of mitosis (prometaphase), the nuclear envelope breaks down and nuclear pore complexes disassemble. This enables microtubules of the spindle to interact with centrosomes [14], [15]. Also at this stage of mitosis a large fraction of cohesin dissociates from chromosome arms.

The N-terminal part of SA2L contains a stretch of basic amino acids 32KNQQGKGTKCCKGDK64 which may represent a mono- or a bipartite NLS. To check whether this sequence is important for nuclear localization of SA2L, we first deleted amino acids 40TKCCKGDK64. However, the protein SA2LΔ40–47 (devoid of this sequence) still localized to the nucleus. We then expanded the deletion by eight amino acids towards the N-terminus and removed 32KNQQGKGTKCCKGDK64. This sequence is important for nuclear localization of SA2L, as we observed that SA2LΔ40–47 was no longer localized to the nucleus.
sequence resembles a classical bipartite NLS, although it does not fully match the consensus and in fact was only identified by manual inspection; the programs used indicated only the putative NLS between positions 40 and 47. The SA2LΔ32–47 protein localized to the cytoplasm, indicating that, contrary to the in silico prediction, the larger bipartite NLS seems to be functional rather than the short monopartite one. In a reciprocal approach, we asked whether the 69 N-terminal amino acids of SA2L can target GFP reporter to the nucleus. The fusion protein SA2L1–69-GFP localized to the nucleus, which confirmed that the analyzed fragment contains a functional autonomous NLS (Figure 3).

To check whether any functional NLS would be sufficient to address SA2 to the nucleus, the short form SA2S (localizing throughout the yeast cell) was fused to a 67-amino acid-long fragment containing a well-characterized NLS of S. cerevisiae histone H2B [25]. Amino acids 29KKTKSTSTDGGK33 of H2B had earlier been shown to direct a reporter protein to the nucleus [26], [27]. The length of the H2B fragment fused to SA2S corresponded almost exactly to the length of the N-terminus of SA2L which was missing in SA2S. However, although we found that the fusion protein H2B1–67-SA2S-GFP was expressed at its expected size, it did not localize to the nucleus (Figure 4). This suggests that the import of SA2 to the nucleus may require a specific NLS, such as the N-terminal one present in SA2L.

Since the SA1 protein was also nuclear in yeast, we asked whether the putative 19-amino acid-long bipartite NLS present in the N-terminal non-conserved part of this protein was responsible. The sequence 34KRKRGRPGRPPSTNKKPRK53 is specific to SA1 and is not homologous to the identified functional NLS32–47 of SA2L. Deleting K34–K53 resulted in the localization of SA1–71-GFP throughout the yeast cell, indicating that, contrary to the in silico prediction, the larger bipartite NLS seems to be functional rather than the short monopartite one. In a reciprocal approach, we asked whether the 69 N-terminal amino acids of SA2L can target GFP reporter to the nucleus. The fusion protein SA2L1–69-GFP localized to the nucleus, which confirmed that it contained a functional NLS (Figure 5).

Thus, although the presence of the SA2S protein in the cytoplasm could simply be due to it never being imported to the nucleus because of the lack of the N-terminal NLS that is present in SA2L, we also considered the possibility of this protein actually shuttling between the nucleus and the cytoplasm due to the co-existence of its other putative NLS motifs and NES motifs.

Functional analysis of putative NES in SA proteins

The presence of numerous putative NLS and NES signals suggested that the actual intracellular localization of the SA proteins could be determined by the balance between the two types of localization signals. To check whether the putative NES were functional we used two approaches, both basing on the fact that the NES identified were of the Crm1-dependent kind. Crm1 is an exportin well conserved between yeast and humans [28]. First, we treated yeast cells expressing SA1 or SA2 variants devoid of their respective N-terminal NLS, characterized above, with leptomycin B (LMB), an inhibitor of certain Crm1 variants [29]. Since Crm1 of standard laboratory yeast strains is insensitive to LMB, we used for these experiments strain CRM1–T339C (MNY8) which bears a leptomycin B-sensitive version of Crm1 [30]. To quantitate the nuclear/cytoplasmic localization of SA1Δ34–53 and SA2S fused with GFP in a population of cells, at least 100 cells were scored according to their fluorescence localization as predominantly cytoplasmic or predominantly nuclear. We found that the addition of LMB to a final concentration of 100 ng/ml to cells in logarithmic phase of growth caused a clear-cut shift of GFP fluorescence to the nucleus in 86% of cells expressing SA2S-GFP (Figure 6A, right), but it did not affect the cytoplasmic localization.

Figure 1. Human proteins SA1 and SA2L expressed in S. cerevisiae differ in subcellular localization. (A) Schematic representation of GFP-fused SA proteins. cDNA from Homo sapiens encoding SA1, SA2L or SA2S was 3’-fused to GFP-encoding sequence, generating VLDL linker. (B) All SA-GFP proteins have predicted molecular weights. Diploid yeast strain irr1Δ/irr1D (lacking one copy of Irr1-encoding gene) was transformed with centromeric plasmid pUG35 bearing hybrid genes encoding proteins shown in (A). Yeast were grown to mid-exponential phase in selective medium. Immunoblots of whole-cell extracts were probed with anti-GFP antibodies. Aliquots of 100 μg of protein/lane were resolved by SDS/8% PAGE. Lane WT: yeast strain without a plasmid, lane GFP: same strain bearing pUG35, lane SA1-GFP: the same strain with plasmid bearing SA1-GFP, lane SA2L-GFP: as before but plasmid bearing SA2L-GFP. (C) Microscopic images of yeast cells expressing SA1-GFP, SA2L-GFP or SA2S-GFP fusion proteins. DNA was stained with DAPI, GFP represents fluorescence of fusion proteins, VIS – transmitted light. The middle panel is a composite of two fields from a single experiment but photographed as separate images, as marked. doi:10.1371/journal.pone.0038740.g001
of SA1Δ34-53-GFP (not shown). As expected, LMB had no effect on localization of SA2S-GFP in cells expressing wild type Crm1 protein insensitive to LMB (Figure 6A, left). To confirm the Crm1-dependent export of SA2S we used another approach. The plasmid encoding SA2S-GFP was introduced into yeast strain ABL11 bearing a thermo-sensitive crm1-1 allele [31]. Transferring of these cells from 30°C to 37°C caused a nuclear shift of the fusion protein in 91% of the cells of an unsynchronized culture (Figure 6B, right). As expected, a shift from 30°C to 37°C failed to affect the nuclear localization of SA2S-GFP in cells expressing the temperature-insensitive wild type Crm1 protein (Figure 6B, left). Figure 6C summarizes those results.

The above results indicated that: a. the SA2 protein devoid of the first NLS (SA2S) loses its nuclear localization because the protein is efficiently exported from the nucleus in a Crm1-dependent manner, which logically requires that b. a functional NLS must still be present in SA2S; and c. the SA1 protein deprived of its first NLS is no longer capable of entering the nucleus.

When SA2LΔ32-47-GFP was studied, the results were qualitatively similar to those obtained for SA2S-GFP, albeit less clear-cut (not shown). First, LMB caused the GFP signal to become predominantly nuclear in only 32% of cells, suggesting additional Crm1-independent nuclear export by an unidentified NES. Second, the ABL11 cells expressing SA2LΔ32–47-GFP became extremely temperature-sensitive and showed increased cell membrane/cell wall fragility when shifted to a higher temperature, resulting in a 20% loss of viability. Nevertheless, nuclear localization of the GFP signal was also observed in 29% of such cells.

When the putative NES of SA1 and SA2 are compared, one notices that in addition to signals common to these two proteins, SA2 contains two signals that are not conserved in SA1. We reasoned that the NES which are found in both SA2 and SA1 are likely to be non-functional since SA1 seems not to be exported. We therefore focused our attention on the two NES unique to SA2.

The two SA2-specific NES (see Figure 2) comprise sequences numbered 689LIRLKKQMRV699 and 953LEKFMTQMSL964 in SA2S. The numbering in SA2L is ABL11 bearing a thermo-sensitive crm1-1 allele [31]. Transferring of these cells from 30°C to 37°C caused a nuclear shift of the fusion protein in 91% of the cells of an unsynchronized culture (Figure 6B, right). As expected, a shift from 30°C to 37°C failed to affect the nuclear localization of SA2S-GFP in cells expressing the temperature-insensitive wild type Crm1 protein (Figure 6B, left). Figure 6C summarizes those results.

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These sequences constitute putative signals for Crm1p-dependent export, the consensus of which is \( W_{X2-3}W_{X2-3}W_{X}W \), where \( W \) represents L, I, V, F or M and \( X \) – any amino acid [32]. The corresponding sequences in SA1 deviate from the consensus. Below we show an alignment of the first of the SA2-specific NES with the corresponding sequence from SA1. To verify the functionality of the predicted NES we first disrupted the original SA2S sequence 689\text{LLRLKK}QMRV\text{699} by substituting V\text{699} with S (both underlined), in this manner mirroring the (predicted to be nonfunctional) SA1 sequence.

\[
\Phi_X_{2-3}\Phi_X_{2-3}\Phi_X\Phi\text{ consensus} \\
\Phi_X_{2-3}\Phi_X_{2-3}\Phi_X\Phi
\]

We found that the substitution V\text{699}S in SA2S shifted the GFP fluorescence to the nucleus in 30% of cells (micrographs not shown, but data included in Figure 7B). This experiment apparently confirmed the functionality of the original NES signal.

However, we found that the plasmid expressing the mutated V\text{699}S SA2S protein also carried a spontaneous single mutation C\text{450}G leading to the substitution S\text{150}R. This region of SA2S does not carry recognizable NLS or NES-like motifs. Despite various experimental approaches taken we were unable to propagate in bacteria a plasmid encoding the protein with the V\text{699}S substitution alone. Thus, we cannot exclude the possibility that the substitution S\text{150}R could also have affected the SA2S localization.

In the second region of interest two putative nested signals for Crm1-NES are predicted. The shorter one is:

\[
\Phi_X_{2-3}\Phi_X_{2-3}\Phi_X\Phi
\]

and the longer one:

\[
\Phi_X_{2-3}\Phi_X_{2-3}\Phi_X\Phi
\]

In the shorter track we substituted F\text{960} (underlined) in SA2S with E. The rationale for this substitution was the same as it was with the first signal – in SA1 glutamic acid is present in the corresponding position. The SA2S-F\text{960}E-GFP localized to the nucleus in 100% of cells, confirming functionality of this NES (Figure 7).

Taken together, the above results suggested that both SA2-specific NES are required for an effective export, albeit the former one (689–699) seems to be less important.

Verification of functionality of SA NLS and NES in HeLa cells

To check if the findings derived from yeast also hold true in mammalian cells we used HeLa cells transiently transfected with plasmids bearing SA1-GFP, SA2S-GFP or SA2L-GFP hybrid genes, and their deletion mutants devoid of sequences encoding putative NLS signals. Figure 8A depicts all constructs which will be discussed below.
All three GFP fusion proteins with wild type SA had nuclear localization (Figure 8B). This result was different from that obtained in yeast where SA2S-GFP was distributed throughout the cell. Subsequently, we introduced a plasmid encoding SA1Δ34–53 protein (devoid of the N-terminal NLS) and found that the protein was distributed throughout the cell, predominantly in the cytoplasm (Figure 8C, upper panel), which confirmed that the same signal is

Figure 6. SA2S shuttles between nucleus and cytoplasm in yeast cells. (A) Subcellular localization of SA2S-GFP was analyzed after addition of LMB (Crm1p inhibitor) to 40 ng/ml to cells in logarithmic phase of growth. Strain CRM1-T539C bears LMB-sensitive version of Crm1p. Fourth column shows a composite of two fields from a single experiment but photographed as separate images, as marked. (B) Localization of SA2S-GFP protein was analyzed in thermo-sensitive crm1-1 mutant. Transfer of cells grown at 30°C to 37°C for 30 minutes caused nuclear shift of the fusion protein in 100% of cells. Third and fourth columns show a composite of two fields from a single experiment, as marked. On the right in (A) and (B) control experiments in wild-type yeast are shown. DNA was stained with DAPI, GFP represents fluorescence of fusion proteins, VIS – transmitted light. (C) Frequencies of cells localized predominantly to the cytoplasm (black) or to the nucleus (gray) in strains bearing CRM1-T539C (LMB-sensitive) or crm1-1 (thermo-sensitive) versions of Crm1p, following LMB treatment or temperature shift, respectively. MN47 and ABL10 are corresponding control strains bearing wild type CRM1 gene, subjected to the same treatments.
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necessary for the nuclear import of SA1 both in S. cerevisiae and in HeLa cells.

The lack of a difference of the cellular localization between SA2L and SA2S indicated that the N-terminal NLS localized between K32 and K47 of SA2L is not necessary in human cells, although it was both necessary and sufficient in yeast. The unaffected nuclear localization of the SA2LΔ32–47 protein, devoid of this N-terminal signal (Figure 8G, lower panel), confirmed that supposition and indicated that another NLS

directs SA2 to the nucleus of HeLa cells. Since in SA1 the only functional NLS was the N-terminal one, the SA2 signals shared with SA1 were unlikely to be functional. We therefore focused on three NLS localized in the C-terminal part of SA2 since one of them (P1129-S1137, numbering for SA2L) was unique to SA2 and two others (R1071-V1084 and P1199-E1206) were similar to but not identical in the amino acid sequences with the respective signals present in SA1. Deletion of the C-terminal 161 amino acids of SA2, comprising all three NLS, resulted in the protein named SA2LΔC161. The truncated protein had exclusively cytoplasmic localization (Figure 8D, upper panel) indicating that the NLS necessary for the import of SA2 to the nucleus (one of the three putative ones or their combination) is localized at the C-terminus.

To pinpoint the C-terminal NLS actually responsible for nuclear localization of SA2 we first deleted amino acids R1071 through S1140, encompassing two of the three C-terminal NLS. This protein still localized to the nucleus, indicating that the C-terminal-most signal (P1199-E1206 in intact SA2L) is sufficient for nuclear addressing. In a reciprocal experiment, we deleted amino acids L199–1206 encompassing only that NLS. Unexpectedly, that protein was also present in the nucleus. This indicates that the nuclear localization of SA2L is executed by redundant signalings in the C-terminal part, of which the first and/or second, or the third, suffice for the addressing. Our attempt to differentiate the role of signals R1071-V1084 and P1199-S1137 (first and second, respectively) failed since the deletion of the latter NLS produced an apparently unstable protein and despite several attempts such a protein could not be detected.

Subsequently, we attempted to verify whether the SA2LΔC161 protein is extranuclear because it never gets imported to the nucleus or because its export from the nucleus predominants over nuclear import weakened by the absence of the C-terminal import signals. The NES signal L1022-L1033, which was functional in yeast, is intact in SA2LΔC161. However, treatment of cells expressing SA2LΔC161-GFP with LMB did not cause a shift of the GFP fluorescence to the nucleus (not shown). Modifications of the original LMB protocol for mammalian cells [33] by increasing the dose of LMB or duration of the treatment did not change the results. This indicated that SA2L protein devoid of the C-terminal part is not imported to the nucleus.

After showing that the redundant NLS functional in HeLa cells are located in the C-terminal part of SA2, we returned to the yeast system. We checked whether the protein SA2SΔC161 (short form, devoid of the N-terminal NLS and lacking the C-terminus) can shuttle between the nucleus and the cytoplasm by performing experiments with leptomycin B. The SA2 variant was, as expected, distributed throughout the cell without LMB treatment and that localization was not altered upon blocking of Crm1-dependent export with LMB (results not shown). This behaviour indicates that the second, weaker NLS functional in yeast that was responsible for the nuclear localization of SA2S in the presence of LMB (see “Functional analysis of putative NES in SA proteins” above) was located in the C-terminal 161 amino acids. Thus, the NLS that were functional in HeLa cells were also recognized as secondary NLS in yeast.

**Discussion**

Protein shuttling between the nucleus and the cytoplasm is controlled by nuclear localization (NLS) and nuclear export sequences (NES) that bind directly, or through adaptor proteins, to specific karyopherins that allow selective and directional passage through the nuclear pore complex [reviewed in [34–36]]. The presence of both types of “address tags” in a single protein allows
modulation of its subcellular localization by largely unstudied mechanisms. Such mechanisms, however, are fundamental to cell functioning since the potential interactors of any given protein are different in the nucleus and cytoplasm, and many proteins shuttle between the two compartments in a cell-cycle- or signal-dependent manner.

_S. cerevisiae_ can be used as a representative model to study the nucleus/ cytosol exchange because the nuclear transport pathways are very highly conserved among lower and higher eukaryotes [37],[38]. Of the fourteen karyopherin family members identified in _S. cerevisiae_, ten have human homologs [39]. Examples of proteins with multiple NLSs have been described, some of which are served by different importin-β (karyopherins that facilitate the import of proteins into the nucleus) family members [40], for review: [41], but in general, little is known about how each import machinery and are necessary to direct these proteins to the nucleus and sufficient to confer nuclear localization on GFP. Affinity capture and mass spectrometry analysis has identified karyopherin Kap123 as an interactor of Irr1, the yeast homologue of SA [42]. It is likely that this karyopherin was also responsible for the nuclear localization of SA1 and SA2.

Since deletion of the N-terminal NLS caused retention of the majority of the SA1 protein in the cytoplasm – the functionality of the other NLS predicted in this protein remains unclear. On the other hand, the SA2S protein which does not contain the N-terminal stretch of amino acids, apparently required for addressing its longer variant SA2L to the nucleus, and also SA2LΔ32–47, can still accumulate in the nucleus when their Crm1p-dependent export from the nucleus is prevented by leptomycin B or a temperature shift of the temperature-sensitive _crm1-1_ strain. This indicates that other NLS signals, besides the N-terminal one, may also be functional in SA2. By comparison with the SA1 protein, in which apparently only the N-terminal NLS is functional, the most likely candidate for the additional functional NLS in SA2 is the penultimate one, since it is the only NLS unique to SA2 apart from the N-terminal one. However, when we deleted this penultimate NLS in SA2S, the mutant protein could still enter the nucleus upon LMB treatment or upon temperature shift of the _crm1-1_ strain (results not shown). This result shows that, contrary to expectations, some other putative NLS must be functional in SA2.

By expressing the truncated SA2S variant we also found evidence for a previously unrecognized Crm1p-dependent export.

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**Table 1. Plasmids used in this study.**

| Plasmid | Relevant plasmid genotype | Reference |
|---------|--------------------------|-----------|
| pUChSA-1 | pUC19-SA1cDNA | J-L. Barbero, Centro de Investigaciones Biologicas (CSIC), Madrid, Spain |
| pUChSA-2 | pUC18-SA2cDNA | as above |
| pUG35 | PMET25-MCS-GFP-TCYC, URA3 CEN | J. H. Hegemann, Heinrich-Heine-Universitat, Dusseldorf, Germany |
| pGouta | PSGAA1-1-67NLS-GFP-TCyC URA3 2 μ | based on pRS426 (Clontech) |
| pPK1 | PMET25-SA1-GFP-TCYC, URA3 CEN | This study, based on pUG35 |
| pLT1 | PMET25-SA2-GFP-TCYC, URA3 CEN | This study, based on pUG35 |
| pLT1-M1 | PMET25-SA2V558S-GFP-TCYC, URA3 CEN | This study |
| pLT1-M2 | PMET25-SA2F599E-GFP-TCYC, URA3 CEN | This study |
| pLT3 | PMET25-1-67NLS-GFP-TCYC, URA3 CEN | This study |
| pPK2 | PMET25-1-67NLS-SSTAG2-GFP-TCYC, URA3 CEN | This study |
| pSA1 | PCYC-SA1-GFP-SV40 Late poly(A) | based on PC.neo (Promega) |
| pSA2L | PCYC-SA2-L-GFP-SV40 Late poly(A) | as above |
| pSA2S | PCYC-SA2S-GFP-SV40 Late poly(A) | as above |
| pSA1Δ34–53 | PCYC-SA1.K34-S3K-GFP-SV40 Late poly(A) | as above |
| pSA2LΔ32–47 | PCYC-SA2.K32-47K-GFP-SV40 Late poly(A) | as above |
| pSA2LΔC161 | PCYC-SA2.I161-GFP-SV40 Late poly(A) | as above |
| pSA2LΔ1107–1140 | PCYC-SA2.I1071-S1140-GFP-SV40 Late poly(A) | as above |
| pSA2Δ1199–1206 | PCYC-SA2.P1199-E1206 -GFP-SV40 Late poly(A) | as above |

Abbreviations for description of plasmids: CEN, centromeric; 2 μ, episomal; MCS, multiple cloning site.

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**Figure 8. NLS of SA1 identified in yeast is the same in HeLa cells but SA2 is targeted to the nucleus of HeLa by C-terminally localized NLS.** (A) Schematic representation of HeLa cells expressing SA1-GFP and SA2-GFP and their deletion mutants. Arrows indicate localization of NLS discussed in the text. Grey color indicates GFP fluorescence. (B) HeLa cells expressing SA1-, SA2L- and SA2S-GFP fusion proteins. (C) HeLa cells expressing SA1-GFP and SA2L-GFP devoid of N-terminal NLS 34–53 and 32–47, respectively. (D) HeLa cells expressing SA2L protein devoid of 161 C-terminal amino acids (upper panel), C-terminal NLS 1071–1140 (middle panel), C-terminal NLS 1199–1206 (lower panel). GFP represents fluorescence of fusion proteins, DNA was stained with DAPI.

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of SA2 cohesin from the nucleus. Human cohesins are believed to be localized to the nucleus until the G2/M transition, when the nuclear envelope is disassembled. The envelope re-forms in the telophase, which is coincident with the reassociation of cohesin with chromosomes. No data indicating cohesin shuttling between nucleus and cytoplasm have been published until now. The closed mitosis of S. cerevisiae used in this study facilitated the observation of the possibility of the SA2 protein moving between the nucleus and the cytoplasm. Since S. cerevisiae cells have many elements of the meiotic division common with mammalian cells, conclusions drawn from yeast research can be applied to higher eukaryotes (where a Crm1p orthologue is known to function) [31]. Such evidence an essential role of the C-terminus in SA2 trafficking in HeLa cells. This region of 161 amino acids comprises three putative NLS acting redundantly: the presence of either the C-terminal one or the first plus the middle one was required for nuclear import. In the SA2 C-terminus numerous mitosis-specific phosphorylation sites have been identified [50] essential for unloading of cohesin from chromosome arms during early mitosis. Since some of those sites are in close proximity of the three C-terminal NLS, one is tempted to speculate on the possible effects of the phosphorylation on the functioning of the adjacent import signals. Our data predict a very complex mechanism of SA2 nuclear import requiring further detailed studies. We assume that, similarly to yeast, also in human cells SA2 may be actively exported from the nucleus, although mechanisms that provoke such nucleocytoplasmic shuttling remain unknown. Since during meiotic division a specific SA paralogue, SA3, replaces most SA1 and SA2 molecules [51], one cellular event that could require the SA1 and SA2 proteins to leave the nucleus could be the switch from mitotic to meiotic division. The mechanism of Crm1p-dependent export has been described for another protein involved in chromosome segregation in human cells – separase. The nuclear exclusion of separase has been postulated to prevent cohesin cleavage in interphase cells [52]. Thus, shuttling between the nucleus and the cytoplasm constitutes an important regulatory mechanism for other multifunctional proteins involved in DNA repair and maintenance of genetic stability [53–56].

Materials and Methods

Strains, growth conditions and genetic procedures

Escherichia coli XLI-Blue MRF (Stratagene, Saint Quentin en Yvelines, France) was used for molecular manipulations. All S. cerevisiae strains used in this study were derivatives of W303. Strain irr1A/IRR1 was described in Cena et al. [21]. ALB11, bearing temperature-sensitive Crm1p, and the control strain ALB10 [57] were provided by Dr. Anita Hopper, Ohio State University, Columbus, OH, USA. Strains MN Y8 (CRM1/T339C), bearing leptomycin B-sensitive version of Crm1p, and the control MNY7/CRAH1 [30] were provided by Dr. Michael Rosbash, Brandeis University, Waltham, MA, USA. Yeast growth and transformation followed standard procedures [25]. To study the effects of Crm1p on localization of human SA proteins, plasmids (described further) bearing cDNA encoding SA1 protein or variants of SA2 fused to the N-terminus of GFP were transformed into crm1-1/stop1-1 (ABL11) and CRM1/XPO1 (ABL10) yeast. The crm1-1/stop1-1 allele supports ABL11 growth at room temperature but not at 37°C [31]. Strains were grown overnight to log phase at 30°C in selective minimal medium. Cultures were divided into halves and incubated at 37°C for 30 min or allowed to continue growing at 30°C. After 30 min cells were fixed with 4% (w/v) formaldehyde and subjected to fluorescence microscopy. For leptomycin B (LMB) treatment the same plasmids were transformed into CRM1/T339C and CRM1 strains. As above, strains were grown overnight, cultures were divided and treated with LMB (LC Laboratories, Woburn, MA, USA, cat. No. L-6100) at 40 ng per ml of medium. Sixty minutes after LMB treatment, cells were collected, fixed with 4% formaldehyde and subjected to fluorescence microscopy.

HeLa cell culture

HeLa cells (European Cell Culture Collection, catalogue no. 93021013) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were incubated in polystyrene flasks (Sarstedt) in 5% CO2-balanced air at 37°C. All cell culture reagents were from Gibco/Life Technologies.

Transient cell transfection

After reaching 100% confluence, cells were seeded on collagen-coated glass coverslips (placed in standard six-well plates) and grown in culture medium for 20–30 hours until 40–60% confluence. Cells were then transfected using the FuGENE reagent (Roche Diagnostics) according to manufacturer’s instructions. For maximal transfection rates, FuGENE was mixed with plasmid DNA at a 7:2 (μg/ml) ratio. In some experiments, leptomycin B was added to the medium (final concentration of 6 ng per ml, unless stated otherwise). The effect of LMB was studied at different intervals from 20 minutes to 18 hours. At 24 hours after transfection, cells were fixed in 4% paraformaldehyde (Sigma) for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 (Sigma) and mounted in SlowFade (Invitrogen) and 0.01 mg/ml DAPI (4’, 6-diamino-2-phenylindole dichloride, Sigma).

Plasmid construction

Plasmids listed in Table 1 were constructed by standard methods. All PCR products were sequenced after cloning. cDNAs serving as templates for amplification of SA1 gene (EMBL/GenBank Accession Number Z75330.1, NM_005862) and SA2 short splice variant (EMBL/GenBank Accession Number Z75331) were obtained from Dr. Jose’-Luis Barbero, Dpto. Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CSIC), Madrid, Spain. SA2 variant 4 (EMBL/GenBank Accession Number NM_006603.3 or NM_006603.4) was purchased from OriGene. To create SA1 and SA2 fusions to GFP, PCR-generated fragments were inserted into the centromeric plasmid pUG35 (kindly provided by Dr. J. H. Hegemann, Heinrichs-Heine-Universität, Düsseldorf, Germany) bearing S. cerevisiae MET25
promoter and a GFP-encoding sequence. To create SA2 fusion to nuclear localization sequence (NLS) of S. cerevisiae histone H2B (codons 1–67) the NLS-encoding fragment was PCR-amplified using pGoutB plasmid [56] as a template. To fuse SA1 and SA2 fragments containing putative NLS with GFP the 3-terminal 213-bp fragment of SA1 or PCR-amplified, introduced into pUG35 and verified by sequencing. In general, plasmids bearing SA1 genes propagate poorly in E. coli. We also noticed spontaneous point mutations occurring in SA1 genes irrespective of the bacterial strain used for propagation. Thus, careful DNA sequencing was done after each manipulation.

Site-directed mutagenesis

Single mutations causing substitutions V698S and F959E of SA2 were generated by overlap extension PCR [38] with pairs of internal complementary oligonucleotides for the desired mutation (F-forward and R-reverse) and two external (E) oligonucleotides (Table S1). The SA2 cDNA in pUC19 was used as the template in the first round of PCR. Both mutant alleles were fully sequenced to verify the presence of desired mutation.

Western blotting

To visualize chimeric GFP-tagged proteins on Western blots, protein samples (100 µg/lane) were subjected to 8% SDS-PAGE. Electrophoresis was followed by blotting onto Hybond-C extra membrane, probing with an anti-GFP antibody (Axxis antibody Living Colors AB, Becton Dickinson), anti-rabbit alkaline phosphatase-conjugated secondary antibody (Promega) and development with CDP-Star (Roche) or Western Blue Stabilized phosphatase-conjugated secondary antibody (Promega) and development with CDP-Star (Roche) or Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

Fluorescence microscopy

Yeast cells were observed and images were taken using a Nikon Eclipse E800 fluorescence microscope with a 100× objective. GFP-fusion proteins were visualized in liquid-grown cells fixed with 4% formaldehyde for 20 min. DAPI was used to stain DNA. To estimate the percentage of yeast cells with a given SA-GFP localization, at least 100 cells were analyzed. For transfected HeLa cells an IX71 Olympus fluorescence microscope was used to analyze the distribution of GFP-fused proteins. For each protein variant tested, at least 100 transfected (GFP-expressing) cells were analyzed in duplicate in two independent transfection experiments.

Supporting Information

Table S1 Oligonucleotides used in construction of SA2 mutants by site-directed mutagenesis.

(DOC)

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Author Contributions

Conceived and designed the experiments: AK. Performed the experiments: LT PK MM MJ. Analyzed the data: AK JF MM DP. Contributed reagents/materials/analysis tools: AK MM DP. Wrote the paper: AK JF.
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