β1-chimaerin belongs to the chimaerin family of GTPase-activating proteins (GAPs) and is encoded by the CHN2 gene, which also encodes the β2- and β3-chimaerin isoforms. All chimaerin isoforms have a C1 domain that binds diacylglycerol as well as tumor-promoting phorbol esters and a catalytic GAP domain that inactivates the small GTPase Rac. Nuclear Rac has emerged as a key regulator of various cell functions, including cell division, and has a pathological role by promoting tumorigenesis and metastasis. However, how nuclear Rac is regulated has not been fully addressed. Here, using several approaches, including siRNA-mediated gene silencing, confocal microscopy, and subcellular fractionation, we identified a nuclear variant of β1-chimaerin, β1-Δ7p-chimaerin, that participates in the regulation of nuclear Rac1. We show that β1-Δ7p-chimaerin is a truncated variant generated by alternative splicing at a cryptic splice site in exon 7. We found that, unlike other chimaerin isoforms, β1-Δ7p-chimaerin lacks a functional C1 domain and is not regulated by diacylglycerol. We found that β1-Δ7p-chimaerin localizes to the nucleus via a nuclear localization signal in its N terminus. We also identified a key nuclear export signal in β1-chimaerin that is absent in β1-Δ7p-chimaerin, causing nuclear retention of this truncated variant. Functionally, analyses revealed that β1-Δ7p-chimaerin inactivates nuclear Rac and negatively regulates the cell cycle. Our results provide important insights into the diversity of chimaerin Rac-GAP regulation and function and highlight a potential mechanism of nuclear Rac inactivation that may play significant roles in pathologies such as cancer.

Chimaerins are a family of GTPase-activating proteins (GAPs) that negatively regulate Rac, a small GTPase that plays important roles in control of cell morphology and locomotion in normal and cancer cells. β1-chimaerin is a member of the chimaerin family that is coded by CHN2, a gene that also codes for β2-chimaerin and the recently identified β3-chimaerin isoform (1–3). These isoforms are generated from alternative transcription start sites located in different promoter regions (2). Deregulation of the CHN2 gene has been associated with a number of human pathologies, including mental disorders (4, 5) and cancers such as glioblastoma, T cell lymphoma, and breast cancer (6–9). This has been mainly attributed to down-regulation of the ubiquitously expressed β2-chimaerin, an isoform with known roles in control of cell migration (10, 11), adhesion (12, 13), proliferation (7), axonal pruning (14), and T cell activation (15).

Like most small GTPases, Rac functions as a molecular switch, cycling between an active state (GTP-bound form) and an inactive state (GDP-bound form). This cycle is controlled by the action of three types of regulatory proteins: GAPs (such as chimaerins), which inactivate Rac by accelerating GTP hydrolysis; guanine nucleotide exchange factors (GEFs), which activate Rac by promoting GDP/GTP exchange; and GDP dissociation inhibitors, which bind to Rac-GDP and maintain this inactive GTPase in the cytoplasm (16). GEFs and GAPs not only regulate the guanine nucleotide binding cycle of Rac but also contribute to its spatial control, which is key to fine-tune Rac signaling outputs (17). The multidomain structure of Rac-GEFs and Rac-GAPs facilitates specific interactions with proteins.

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This article contains Figs. S1–S3.

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and lipids that ultimately result in Rac activation at discrete cellular locations in response to specific signaling inputs (16, 17). For example, chimaerins have a regulatory C1 domain that binds the lipid second messenger diacylglycerol (DAG) and DAG mimetics such as phorbol esters (18). Relocalization of chimaerins to the cell membrane by DAG is a key step for their association with active Rac to promote its inactivation (19, 20). In the case of β2-chimaerin, plasma membrane association also involves interaction with the adaptor protein Nck1 via an atypical proline-rich domain adjacent to the C1 domain (21). Chimaerins also relocalize to internal membranes in response to phorbol esters and display perinuclear (Golgi) localization upon stimulation (18, 22).

Although most established Rac functions emanate from active Rac at the plasma membrane (23, 24), it is now recognized that Rac located in other intracellular compartments is also functionally relevant. For example, in the Golgi, Rac localizes with the GAP protein OCRL1 and regulates actin polymerization and vesicle trafficking (25, 26). Mitochondrial Rac1 participates in superoxide production and regulation of cell death (27). Rac1 has also been reported to relocalize to the nucleus, which depends on a functional nuclear localization signal (NLS) sequence that interacts with the nuclear import receptor karyopherin α2 (KPNA2) (28, 29). Interestingly, nuclear localization is specific to Rac1 because the closely related GTPases Rac2 and Rac3 lack an NLS motif (30). Furthermore, nuclear entry of Rac1 is highly conserved in evolution because the NLS sequence is present in a broad range of Rac1 orthologs from nematodes to humans (30) and has also been described in lower organisms such as fungi (31). Export of Rac1 from the nucleus involves the synergistic action of two nuclear export signals (NESs) that may utilize canonical nuclear export routes (32). From a functional standpoint, nuclear Rac has been implicated in regulation of the nuclear actin cytoskeleton and has been associated with cell division, nuclear plasticity, nuclear transport, and transcription of ribosomal DNA (32–34). Importantly, studies have revealed that deregulation of Rac nucleocytoplasmic shuttling is causally associated with pathologies such as cancer (9, 35). Despite remarkable evidence of a nuclear Rac1 pool (25, 31, 33, 36), it remains to be fully established how Rac1 activity is regulated in the nucleus. Identification of functional Rac-GEFs in the nucleus (34, 35) suggests a cycling mechanism of Rac in this compartment.

In this study, we report the identification and characterization of a novel chimaerin variant, β1-Δ7p-chimaerin, that shows prominent nuclear localization. A comprehensive mutagenesis analysis defined the structural determinants responsible for localization of this variant in the nucleus. In addition, we demonstrated that β1-Δ7p-chimaerin acts as a negative regulator of Rac in this cell compartment and identified a functional role of this chimaerin variant in regulation of the cell cycle.

Results

Identification of a β1-chimaerin variant generated by alternative splicing

β1-chimaerin is one of the two main transcripts encoded by the CHN2 gene, and it is generated by an alternative transcription start site located upstream of exon 7 (exon 1 in β1-chimaerin) (1, 2) (Fig. 1A). There is scarce information about β1-chimaerin, which has only been reported in testis (1). To further investigate the expression of this chimaerin isoform, we performed nested PCR in a series of cell lines and tumor samples. This analysis resulted in amplification of transcripts of smaller size than that of the full-length β1-chimaerin transcript. These transcripts were cloned and sequenced. Remarkably, one of these transcripts had a partial deletion of exon 7 (nucleotides 112–246) that does not affect the ORF, giving rise to a shorter variant with an in-frame deletion of 45 amino acids. This variant was named β1-Δ7p-chimaerin (GenBankTM/EBI accession number EU732752.1) (Fig. 1A). The deletion in exon 7 occurs upstream of a GT dinucleotide, which is part of the 5′ splice site consensus sequence typically recognized by the spliceosome at the exon–intron junction (37). Therefore, to evaluate whether β1-Δ7p-chimaerin could be generated by an unknown splicing event of exon 7, we analyzed the exon 7/intron 7 sequences using the NNSsplice predictor (http://www.fruitfly.org/seq_tools/splice.html) (38). This analysis revealed the presence of a 5′ donor splice site with a relatively high score (0.78) located 135 bp upstream of the canonical splice site (Fig. 1B), which indicates that β1-Δ7p-chimaerin is generated by alternative splicing.

Next we evaluated the expression of β1-Δ7p-chimaerin by quantitative real-time PCR using a panel of cDNAs from 48 human tissues and compared it with that of β1-chimaerin. Primers were designed for specific amplification of each chimaerin variant, as shown in Fig. 1C. This analysis revealed that most tissues expressed both chimaerin isoforms, although at moderate levels. We found different patterns of expression in several tissues, with β1-Δ7p-chimaerin more abundant than β1-chimaerin in bone marrow, the brain, lymph nodes, and mammary glands (Fig. 1D). β1-Δ7p-chimaerin was also expressed in several human tumor cell lines, being more abundant in breast and lung tumor cell lines (Fig. S2).

The truncated β1-Δ7p-chimaerin variant localizes into the nucleus

To begin characterizing the β1-Δ7p-chimaerin isoform, we first evaluated its intracellular distribution relative to that of full-length β1-chimaerin (β1-FL). To this end, we generated plasmids for expression of these two proteins. Because of the lack of highly sensitive and specific β1-chimaerin antibodies, we expressed the proteins fused to either FLAG or EGFP tags. The corresponding plasmids were transfected into COS-1 cells, and the proper expression and molecular size of each protein were corroborated by Western blotting (Fig. 2A). Confocal microscopy analysis revealed that most cells (80%) showed β1-FL located only in the cytoplasm (Fig. 2, B and C). In sharp contrast, β1-Δ7p-chimaerin exhibited marked nuclear localization in most cells (98%) and was predominantly nuclear in ~50% of the cells (Fig. 2, B and C). A similar distribution was observed for both FLAG- and EGFP-tagged proteins (Fig. 2B), suggesting that nuclear localization is an intrinsic property of
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Figure 1. Identification of a novel β1-chimaerin variant generated by alternative splicing. A, schematic of the CHN2 gene. Numbers indicate the CHN2 exons. Arrows show the transcription start sites for β2-chimaerin (on exon 1) and β1-chimaerin (on exon 7). The exon arrangements leading to β2-, β1-, and β1-Δ7p-chimaerin are indicated. The sequence exclusive of β1-chimaerin in exon 7 is shown in gray. The shorter exon 7 in β1-Δ7p-chimaerin is represented in red.

B, schematic of splicing of exons 7 and 8 in β1- and β1-Δ7p-chimaerin. Partial sequences of exon 7 and 8 (capital letters) and intron 7 (lowercase letters) are shown. Black box, canonical donor splice site; red box, cryptic alternative donor splice site; green box, acceptor splice site. Predicted scores of the splice donor sites of exon 7 by NNSplice are displayed below each splice site.

C, schematic of the primers for specific amplification of β1-Δ7p- and β1-chimaerin. The specificity of the primers was corroborated by PCR (bottom panel) using expression vectors for β1-Δ7p-chimaerin (β1-Δ7p), β1-chimaerin (β1-FL), or β2-chimaerin (β2-FL) as a template.

D, real-time qPCR analysis of β1-Δ7p- and β1-chimaerin expression in human tissues.
the $\beta_1$-Δ7p-chimaerin spliced variant. The subcellular localization of $\beta_1$-Δ7p-chimaerin was also studied in primary cells. To this end, mouse embryonic fibroblasts (MEFs) were transfected with EGFP-tagged $\beta_1$-Δ7p-chimaerin, and its subcellular distribution was compared with that of transfected $\beta_1$-FL (Fig. S1A). $\beta_1$-Δ7p-chimaerin was also mainly nuclear in these cells, whereas $\beta_1$-FL located mostly in the cytosol.

To further validate the nuclear localization of $\beta_1$-Δ7p-chimaerin, we carried out a subcellular fractionation analysis. Nuclear and cytosolic compartments of COS-1 cells transfected with pEGFP-β1-Δ7p-chimaerin were prepared, and chimaerin expression was analyzed by Western blotting. To rule out nuclear and/or cytoplasmic contamination, cytoplasm- and nucleus-specific controls were included in the immunoblots (tubulin and lamin AC, respectively). In agreement with the confocal microscopy results, $\beta_1$-Δ7p-chimaerin was highly abundant in the nuclear fraction (Fig. 2D).

As mentioned above, $\beta_1$-Δ7p-chimaerin has a 45-amino-acid deletion spanning part of the N-terminal region and the first four amino acids of the C1 domain (Fig. 2E). Typical C1 domains regulate protein association with cell membranes by binding to DAG or DAG mimetics such as phorbol esters (39). Based on the known structural features of DAG-responsive C1 domains (40, 41), the prediction is that the truncated C1
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domain in β1-Δ7p-chimaerin cannot be properly folded to bind ligands. To test this hypothesis, we treated transfected COS-1 cells with phorbol 12-myristate 13-acetate (PMA), which induces relocation of chimaerins from the cytosol to the plasma membrane and the perinuclear region (22, 42). Accordingly, PMA caused translocation of β1-chimaerin mostly to the perinuclear region, as described previously (22). However, no changes in subcellular localization of β1-Δ7p-chimaerin were observed in response to PMA treatment (Fig. 2F), supporting the concept that the truncated C1 domain in β1-Δ7p-chimaerin makes it unresponsive to DAG and phorbol esters. These results revealed that β1-Δ7p-chimaerin is a nuclear chimaerin variant with a nonfunctional C1 domain. Therefore, in contrast to all other known chimaerin isoforms, β1-Δ7p-chimaerin is not regulated via DAG binding to the C1 domain (39).

Identification of an NLS required for nuclear localization of β1-Δ7p-chimaerin

One plausible explanation for the nuclear localization of β1-Δ7p-chimaerin is that the sequence truncation generated an NLS. To test this hypothesis, we analyzed the β1-Δ7p-chimaerin sequence using various NLS predictor programs. Analysis with PSORTII (43) and NLS Tradamus (44) predicted a bipartite NLS (residues 12-28, named NLS-A), characterized by two basic residues, a 10- to 12-amino-acid spacer, and another basic region in which three of five amino acids must be basic (45, 46) (Fig. 3A). A second monopartite NLS that encompasses residues 88-92 (named NLS-B) was also identified with PSORTII and nCNS Mapper (43, 47). This NLS fulfills the criteria of a Pat7 monopartite NLS that consists of a Pro followed within three residues by a segment containing three basic residues of four amino acids (45, 46) (Fig. 3A). To evaluate whether these putative NLSs are functional, we carried out a mutational analysis at the key residues in the NLS consensus sequences (48) (Fig. 3B). The corresponding EGFP-tagged β1-Δ7p-chimaerin NLS mutants were expressed in COS-1 cells (Fig. 3C), and nuclear localization was evaluated by confocal microscopy (Fig. 3D). Remarkably, Ala substitutions in the second cluster of basic residues of the bipartite NLS (residues 26-28, mutant Δ7p-NLS-A1) resulted in a marked change in intracellular localization. Indeed, although WT β1-Δ7p-chimaerin had discernible nuclear localization in nearly every cell, with primary nuclear localization in ~50% of the cells, the bipartite NLS mutant located in the cytoplasm in 34% of cells, with essentially no cells showing primary nuclear localization. Mutation of the first two Lys to Ala in NLS-A (residues 12KK13, mutant Δ7p-NLS-A2) also had a shifting effect toward cytoplasmic localization, although the effect was less pronounced. The combination of the mutations in both clusters of basic amino acids (mutant Δ7p-NLS-A1 + 2) resulted in a slight increase in cytoplasmic localization relative to individual mutant Δ7p-NLS-A1 and mutant Δ7p-NLS-A2, although the effect was not additive, and this double mutant could still be observed in the nucleus in 55% of cells (Fig. 3, D and E). The subcellular localization of this mutant was also studied in MEFs (Fig. S1B). Similar to the results in COS cells, the NLS-A1 + 2 mutation resulted in increased cytoplasmic localization of β1-Δ7p-chimaerin and a concomitant reduction in the primary nuclear localization of this protein.

NLS-B (residues 85PDLKRIKK92) was also examined for a potential role in nuclear import. We generated a mutant in which basic residues at positions 88 and 89 were mutated to Ala (88KR, mutant Δ7p-NLS-B1) as well as a mutant with four basic residues mutated (88KR, 91K)2, mutant Δ7p-NLS-B1 + 2). Unlike NLS-A, mutations in NLS-B had a minimal effect, with only a small increase in cytoplasmatic localization and essentially no changes in the number of cells with primary nuclear localization, resembling WT β1-Δ7p-chimaerin (Fig. 3, D and E). These experiments suggest that the sequence described as NLS-B is not an effective NLS. To further support this conclusion, we generated a mutant β1-Δ7p-chimaerin in which both putative NLS sequences were mutated (mutant Δ7p-NLS-A+B). As predicted from experiments with mutations in individual putative NLSs, the Δ7p-NLS-A+B mutant behaved like the Δ7p-NLS-A1 mutant, with a significant increase in cytosolic localization and essentially no primary nuclear localization. Taken together, these results revealed the N-terminal bipartite NLS as the key domain required for efficient nuclear import of β1-Δ7p-chimaerin.

Although we expected a functional NLS to be a specific feature of β1-Δ7p-chimaerin, this signal is located in the N-terminal region upstream of the amino acid truncation; therefore, it is also present in the β1-chimaerin isoform (Fig. 3A). Furthermore, alignment of the N-terminal β1-chimaerin sequences retrieved from a Blastp search (NCBI) revealed that the bipartite NLS sequence is conserved among different mammal species (Fig. 3F). On the other hand, alignment of human β1- and α1-chimaerins showed that the basic residues of the consensus sequence are not present in α1-chimaerin (Fig. 3G), an indication that this bipartite NLS is a specific feature of β1-chimaerin isoforms that is conserved in mammals.

β1-Δ7p-chimaerin nuclear localization is a consequence of loss of an NES

It is noteworthy that both β1-chimaerin and β1-Δ7p-chimaerin share a NLS, but only β1-Δ7p-chimaerin is predominantly nuclear. One potential scenario is that a signal responsible for the nuclear export of β1-chimaerin is not present in β1-Δ7p-chimaerin. To test this hypothesis, we searched for the presence of NESs in the stretch of amino acids deleted in the β1-Δ7p isoform using the NetNES server (49). This search revealed a motif (residues 45L-F52) that fits the loose consensus for a leucine-type NES (49) (Fig. 4A). Like the NLS, the putative NES is highly conserved in β1-chimaerin from different species (Fig. 4B). To assess the functional relevance of this potential NES, we mutated hydrophobic amino acids known to be critical for NES activity (49, 50). Mutations included single, double, and triple substitutions in Leu45, Leu49, and Leu51 in WT β1-chimaerin (Fig. 4C). The intracellular localization of these mutants was evaluated by confocal microscopy upon expression in COS-1 cells. All mutant proteins were detected as single bands of the expected molecular weight (Fig. 4D). As shown in Fig. 4, E and F, single mutations of the Leu residues in the putative NES had modest or no effects on intracellular localization. On the other hand, double mutation of amino acids Leu49 and Leu51 (mutant
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Figure 3. The N-terminal region of β1- and β1-Δ7p-chimaerins contains a functional NLS. A, predicted NLSs in β1- and β1-Δ7p-chimaerins. Consensus sequences for each type of NLS are shown, and residues fulfilling the consensus criteria are highlighted. B, point mutants generated on the β1-Δ7p-chimaerin potential NLSs (amino acid changes are indicated in bold). C, Western blot analysis of NLS mutants expressed in COS-1 cells. Proteins were detected with anti-GFP antibody. D, representative confocal immunofluorescence images of COS-1 cells transfected with the indicated EGFP-tagged mutants. Scale bars = 20 μm. Nuclei were stained with DAPI. E, subcellular distribution of each mutant was analyzed in at least 100 cells scored in each of three different experiments. The result are given as >Nuclear, Nuc = Cyt, or >Cytoplasmic to indicate the predominant subcellular distribution of each mutant. Results are presented as means ± S.D. F, alignment of the bipartite NLS of β1-chimaerin. Sequences were retrieved from a Blastp search (NCBI) with the human β1-chimaerin sequence (NP_001035025.1) and aligned using ClustalO software. Sequences correspond to the following species: Homo sapiens (NP_001035025.1), Pongo abelii (PNI55618.1), Pan troglodytes (PNJ56018.1), Carlito syrichta (XP_008067179.1), Heterocephalus glaber (XP_012925731.1), Cavia porcellus (XP_003467878.1), Mus musculus (NP_076032.2), Bos taurus (NP_001039428.1), and Odocoileus virginianus texanus (XP_020748415.1). Conserved basic residues are highlighted. G, alignment of the bipartite NLS of human β1-chimaerin (NP_001035025.1), β1-Δ7p-chimaerin (ACF04989.1), and α1-chimaerin (NP_001193531.1).
Figure 4. Loss of an NES results in nuclear accumulation of β1-Δ7p-chimaerin. A, computational analysis predicts one NES in β1-chimaerin that is lost in the β1-Δ7p-chimaerin isoform. The consensus sequence is shown, and residues fulfilling the consensus criteria are highlighted. B, alignment of the NES sequence of β1-chimaerin. Alignment was performed as in Fig. 3F. C, point mutants generated on the β1-chimaerin potential NES (amino acid changes are indicated in bold). D, Western blot analysis of the NES mutants expressed in COS-1 cells. E, representative confocal immunofluorescence images of COS-1 cells transfected with the indicated EGFP-tagged mutants. Scale bars = 20 μm. Nuclei were stained with DAPI. F, subcellular distribution of each mutant was analyzed in at least 100 cells scored in each of three independent experiments. The result are given as >Nuclear, Nuc = Cyt, or >Cytoplasmic to indicate the predominant subcellular distribution of each mutant. Results are presented as means ± S.D.
1-NES L49A, L51A) had a very strong effect on intracellular distribution. Indeed, although the majority of WT 1-chimaerin is localized in the cytoplasm (79% of cells primarily cytoplasmic, 21% of cells with similar nuclear and cytoplasmatic localization, and no cells with exclusive nuclear localization), 1-NES L49A, L51A displays very strong nuclear localization, with more than 90% of cells showing this mutant protein in the nucleus. Mutation of these residues also induced a shift from cytosolic to nuclear distribution of 1-chimaerin in MEFs, although the effect was less pronounced than in COS-1 cells (Fig. S1C). Similar results were observed with the triple mutant 1-NES L45A,L49A,L51A (Fig. 4, E and F). These results show that Leu49 and Leu51 are critical for nuclear export of 1-chimaerin. Altogether, these results provide strong evidence of the presence of a functional NES signal that retains 1-chimaerin in the cytoplasm. The absence of this NES in 1-Δ7p-chimaerin redirects this chimaerin variant to the nucleus.

**1-Δ7p-chimaerin regulates nuclear Rac1**

Several studies have demonstrated the presence of functional Rac1, the effector for chimaerins, in the nucleus (28–34, 36, 51). It is therefore conceivable that 1-Δ7p-chimaerin may regulate Rac1 activity in the nuclear compartment. To test this hypothesis, we first analyzed the effect of ectopically expressing 1-Δ7p-chimaerin on the activation status of nuclear Rac1. Active Rac pulldown assays were performed in nuclear extracts from COS-1 cells expressing EGFP-1-Δ7p-chimaerin or EGFP as a control. As shown in Fig. 5A, the expression of 1-Δ7p-chimaerin reduced nuclear Rac-GTP levels, arguing for a role of this chimaerin isofrom in the control of Rac1 in the nucleus. To further demonstrate this function, we evaluated the effect of down-regulating 1-Δ7p-chimaerin in nuclear Rac activation. To this end, we first performed a search for cell lines with significant expression of this chimaerin isoform. As shown in Fig. S2A, AU565 (human breast cancer) and H358 (human lung cancer) cells showed the highest 1-Δ7p-chimaerin expression and, thus, were chosen for this study. Because 1-Δ7p-chimaerin shares 100% of the sequence with that of 1-chimaerin, we could not generate a specific siRNA for this truncated isoform. Thus, we made use of a validated CHN2 siRNA that recognized a region common to all 1-chimaerin isoforms.
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transcripts. Cells transfected with the CHN2 siRNA showed a 65%–90% reduction on β1-Δ7p-chimaerin levels, as demonstrated by qPCR analysis (Fig. 5, B and C). Down-regulation of β1-Δ7p-chimaerin increased the levels of nuclear Rac-GTP in both cell lines by ~2-fold (Fig. 5, B and C). We discarded an effect of down-regulation of FL β1-chimaerin in this result because this isoform is minor in these cells (Fig. S2B). These results indicate that β1-Δ7p-chimaerin is a bona fide nuclear Rac-GAP.

Nuclear Rac1 is implicated in control of cell cycle progression (33). Thus, we evaluated the effect of β1-Δ7p-chimaerin on the cell cycle. We performed these experiments in cells ectopically expressing β1-Δ7p-chimaerin. We discarded use of silenced cells for these experiments because cell cycle progression could be affected by down-regulation of β2-chimaerin, an isoform with a known role in control of the cell cycle (52). First we analyzed the effect of stable expression of β1-Δ7p-chimaerin in COS-1 cells. To this end, exponentially growing cells were stained with propidium iodide, and DNA content was measured by FACS to determine the cell cycle status. Compared with control cells, expression of β1-Δ7p-chimaerin significantly increased the percentage of cells in S phase (38% of cells expressing EGFP versus 47% in cells expressing EGFP-β1-Δ7p-chimaerin) (Fig. 6A), suggestive of slower progression through this phase. To further evaluate cell cycle progression, cells were treated with hydroxyurea (HU) to block cells at late G1–early S phase, and cell cycle distribution was analyzed after blockade release (Fig. 6B). After HU treatment, most control cells were in G1 or S phases, and only a small fraction (9%) was in G2. However, HU did not fully block the cell cycle in β1-Δ7p–expressing cells because a significant fraction was in G2 phase (~25%). Upon release from HU treatment, both control and β1-Δ7p–expressing cells progressed through S phase to G2 phase, as observed by the increase in S phase population after 3 h and in G2 population after 6 h.

Next we evaluated the effect of β1-Δ7p-chimaerin on expression of the cell cycle markers cyclin D1, cyclin E1, and cyclin B1. Because of the low percentages of EGFP-positive cells we obtained in stable cell lines, for these experiments we used transiently transfected COS-1 cells, which show a similar response to cell cycle progression as stably transfected cells (Fig. 5C). As shown in Fig. 6C, expression of β1-Δ7p-chimaerin resulted in significantly higher expression of cyclin E1 at the initial time points, whereas the levels of cyclin D1 and B1 were not significantly affected. Altogether, these results indicate that the expression of β1-Δ7p-chimaerin led to slower S phase progression, unveiling a role of this chimaerin variant in control of the cell cycle.

Discussion

In this paper, we report the identification of β1-Δ7p-chimaerin, a novel β1-chimaerin variant generated by alternative splicing of the CHN2 gene. β1-Δ7p-chimaerin localizes predominantly in the nucleus because of the presence of an NLS and lack of an NES that is present in β1-chimaerin. We also demonstrated that β1-Δ7p-chimaerin inactivates nuclear Rac and regulates cell cycle progression.

β1-Δ7p-chimaerin is, to our knowledge, the only chimaerin isoform originated by alternative splicing. The first β-chimaerins identified, β1- and β2-chimaerins, although initially considered splice variants (1, 3), are, in fact, generated from alternative transcription start sites on the CHN2 gene (2) The same scenario applies to α1- and α2-chimaerins, whose expression is controlled by two different promoters in the CHN1 gene (53).

Our analysis revealed that the β1-Δ7p-chimaerin transcript is produced by use of a cryptic 5’ donor splice site in exon 7 that obeys the GT-AG rule (37). The mechanisms involved in activation of this cryptic splice site remain unknown. In higher eukaryotes, splicing depends on multiple factors acting in combination to determine splice site selection (54). One of these factors is the presence or absence of splicing regulators, most commonly exon splicing enhancers and exon splicing silencers.

We carried out a bioinformatics analysis of the sequence flanking the canonical and cryptic donor sites in search of these regulatory motifs (Human Splicing Finder; http://www.umd.be/HSF3/) and found a similar pattern of predicted exon splicing enhancers and exon splicing silencers in the vicinity of both sites (data not shown). Although additional studies of these motifs are needed to verify whether they are operative, it may be also possible that other mechanisms contribute to generation of β1-Δ7p-chimaerin. In any case, the wide tissue distribution of this isoform suggests that the cryptic 5’ donor splice is broadly selected by the splicing machinery. It is noteworthy that an acceptor sequence located in exon 7 is used for exon 6–7 splicing of β2-chimaerin, resulting in a shorter exon 7 (2).

The splicing events on exon 7 in the different β-chimaerin isoforms confer unique features to each variant (Fig. 7). Unlike β1- and β2-chimaerins, β1-Δ7p-chimaerin lacks a functional C1 domain, and it is unresponsive to phorbol esters (Fig. 2F). However, in all other chimaerin isoforms, a fully functional C1 domain plays a fundamental role in redistribution from the cytosol to the plasma membrane and endomembranes, where chimaerins associate with active Rac to promote its inactivation (18, 19, 22, 42).

Another unique feature of β1-Δ7p-chimaerin is its prominent nuclear localization. Nuclear entry of proteins is controlled via different mechanisms. Although small proteins can passively diffuse across the nuclear envelope, larger proteins usually require nuclear transporters known as importins, which recognize NLSs (55). Theoretically, because of its size (33 kDa), β1-Δ7p-chimaerin could enter the nucleus by diffusion. However, we observed that the EGFP-tagged version of this protein (>60 kDa) also localizes in the nucleus, which made us hypothesize the existence of NL5s in β1-Δ7p-chimaerin. In our analysis, we identified that an N-terminal bipartite NLS is mainly responsible for directing nuclear entry. Bipartite NLSs consists of two stretches of basic amino acids separated by a linker region. Based on our analysis, the distal basic stretch in the β1-Δ7p-chimaerin’s bipartite NLS has a fundamental role in directing nuclear import.

A puzzling observation was that the functional NLS is not only present in β1-Δ7p-chimaerin but also in the cytosolic β1-chimaerin. It may be possible that the NLS is occluded within the β1-chimaerin structure, preventing its function. Although the tertiary structure of this isoform is not known,
data derived from the crystal structure of β2-chimaerin show that chimaerins are folded into a “closed” conformation that keep them in an inactive state in the cytosol (56). Extensive intramolecular contacts between domains and linker regions in β2-chimaerin leave the C1 and GAP domains buried and inaccessible to DAG and Rac, respectively (56). Most probably, the NLS in β1-chimaerin is not exposed, precluding protein binding to the nuclear transporter, whereas a disorganized C1 domain in β1-Δ7p-chimaerin may leave the NLS accessible to the transport machinery. In this model, an intact C1 domain in β1-chimaerin may be the predominant targeting signal, whereas the NLS represents the key localization factor in β1-Δ7p-chimaerin. Protein–protein interactions involving the C1 domain, such as association with the Golgi protein Tmp-21, may also represent key intracellular positional drivers (57), but only for chimaerin variants with intact C1 domains. It is noteworthy that the NLS is specific to β1-chimaerins because it is not present in β2-chimaerin as result of the exon 6–7 splicing, or it is not conserved in the highly homologous α1-chimaerin (Figs. 3G and 7).

Although mainly cytosolic, β1-chimaerin shows some degree of nuclear localization suggesting that the NLS may be actually
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Identification of a chimaerin isoform in the nucleus raised the question of its nuclear function. It is now well-established that Rac1 shuttles between the cytosol and the nucleus. Nuclear pools of Rac1 regulate various cellular functions, such as cell cycle progression, nuclear actin cytoskeleton reorganization, and transcription of ribosomal DNA (28, 32–34). Rac-GEFs responsible for GTP loading, including Tiam1, Dedicator of cytokinesis (DOCK), Vav1, and Ect2, have been identified in the nucleus (34, 59–61). Moreover, Ect2 has been shown to activate nuclear Rac (34). Consistent with the established cycling of Rho GTPases between GTP-bound active and GDP-bound inactive forms, the prediction is that nuclear Rac-GAPs must exist. To date, only MgcRacGAP has been reported in the nucleus, although, in this particular case, it functions as a chaperone for STAT transcription factors rather than a Rac-inactivating protein (62). Our experiments demonstrate that β1-Δ7p-chimaerin is a nuclear GAP because down-regulation of the endogenous protein increases the levels of endogenous Rac-GTP in the nucleus.
nuclear compartment, whereas its ectopic expression inactivates nuclear Rac. We also provide evidence of a role of $\beta_1$-7p-chimaerin in regulation of the cell cycle, as revealed by slower progression through S phase and elevated cyclin E levels in cells ectopically expressing this protein. Because nuclear Rac1 participates in control of cell division, we speculate that this effect of $\beta_1$-7p-chimaerin on the cell cycle is mediated through Rac1 inactivation. Michaelson et al. (33) first demonstrated that the levels of nuclear Rac1 fluctuate during cell cycle progression, with the highest accumulation in late G2. In their study, nucleus-targeted active Rac1 promotes cell division. Thus, inactivation of nuclear Rac1 by ectopic expression of $\beta_1$-7p-chimaerin is consistent with slower progression through the cell cycle. Mechanistically, Rac1 regulates G1/S transition by controlling cyclin D1 transcription, which is accomplished through various mechanisms, including NF-κB–dependent signaling. Because NF-κB can repress cyclin E expression (63, 64), our model is that inhibition of nuclear Rac1 by $\beta_1$-7p-chimaerin results in increased cyclin E expression.

In summary, here we identified $\beta_1$-7p-chimaerin as a new member of the chimaerin family of Rac-GAPs with a unique nuclear localization and function. We describe for the first time the presence of NES and NLS motifs in $\beta_1$-chimaerin isoforms that play fundamental roles in dictating intracellular localization. Given that deregulation of nuclear Rac emerged as an important factor in pathologies such as cancer, it would be interesting to determine whether $\beta_1$-7p-chimaerin contributes to these pathologies.

**Experimental procedures**

**Plasmids and site-directed mutagenesis**

The full-length $\beta_1$-chimaerin and $\beta_1$-7p transcripts were amplified using nested PCR. To this end, RNA was extracted from different human cell lines and tumor tissues using TRIzol, and cDNA was synthesized by RT-PCR using the ImProm-II™ reverse transcription system (Promega). 100 ng of cDNA was used to perform a PCR with the following oligonucleotides: 5′-CTGTGTCAACTTGGATGGTGC-3′ (forward) and 5′-GATGAGCCCCACATGAAA-3′ (reverse). The number of copies in each human tissue sample was calculated with a calibration curve as described previously (66) using serial dilutions of the pCEFL-FLAG-$\beta_1$-7p and pCEFL-FLAG-$\beta_1$-chimaerin plasmids. The expression level of $\beta_1$-7p-chimaerin was also determined in cDNA obtained as indicated before from the following human cell lines: AU565, BT549, MDA-MB-231, and MDA-MB-453 (breast cancer); A549, H358, H1650, and H3122 (lung cancer); Panc1, AsPC1, and MiaPACA (pancreatic cancer); C30, OVO2, OVO3, and SKVO3 (ovarian cancer); DU145, LnCAP, PC3, and PC3-M (prostate cancer); ANC3CA (endometrial cancer); and H295R (adrenal gland carcinoma). Expression in cell lines was analyzed with the TaqMan probe AAGTCCACAGTTCGGAG-CCCA using TaqMan Universal PCR Master Mix (Applied Biosystems) and was normalized to expression of the GAPDH or UBC housekeeping gene.

**Cell culture, transfection, and siRNA interference**

COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. Cells were transfected with FuGENE6 (Roche Molecular Biochemicals) according to the manufacturer’s instructions. COS-1 cells stably expressing EGFP-$\beta_1$-7p-chimaerin or EGFP were generated by transfection of the corresponding pEGFP expression vectors. Forty-eight hours post-transfection, cells were selected with 800 μg/ml Geneticin and sorted by FACS to collect EGFP-positive cells. The percentages of positive cells after two rounds of selection were 14% and 20% of cells expressing EGFP-$\beta_1$-7p-chimaerin and EGFP, respectively. Expression of the corresponding proteins was confirmed by Western blotting.

MEFs were obtained from embryonic day 12.5 mouse embryos as described previously (67) and cultured in the same medium as COS-1 cells. MEFs were transfected with Lipo-fectamine 2000 (Invitrogen) according to the manufacturer’s instructions. AU565 and H358 cells were used for siRNA interference experiments. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. siRNA to the CHN2 mRNA was purchased from Sigma (MISSION esiRNA, EHU071321). An siRNA targeting firefly luciferase was used as a negative control (Sigma, EHUF-LUC). siRNAs were transfected using Lipo-fectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were grown for 2 days after transfection and harvested for active Rac determination as described below.

**Confocal microscopy**

COS-1 cells and MEFs were grown on glass coverslips and transfected as indicated above. After 24–48 h, cells were washed and fixed with formaldehyde. For staining of FLAG-tagged $\beta_1$-7p-chimaerin, cells were permeabilized in 0.2% Triton X-100 for 10 min and incubated in blocking solution containing 1% BSA in PBS. Cells were then labeled with anti-
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FLAG M2 (Sigma), followed by incubation with anti-mouse Alexa Fluor 488 secondary antibody (Molecular Probes). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) (1 μg/ml). Cells were imaged using a laser-scanning confocal microscope (Leica TCS SP5), and confocal micrographs were processed with ImageJ (National Institutes of Health).

Western blot analysis

Cells were lysed in a buffer containing 20 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM DTT, 5 mM NaF, and protease inhibitors (Complete, Roche Molecular Biochemicals). The lysate was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was collected. Protein concentration in cell lysates was determined using Bio-Rad protein assay dye reagent. Lysates were mixed with Laemmli sample buffer, and equivalent amounts of protein were resolved by SDS-PAGE and processed by immunoblotting analysis as described previously (68, 69). The following primary antibodies were used: anti-GFP (Covance, MMs-118P), anti-FLAG M2 (Sigma-Aldrich, F3165), anti-cyclin D1 (Cell Signaling Technology, 92G2), anti-cyclin E1 (Cell Signaling Technology, HE12), anti-cyclin B1 (Cell Signaling Technology, 4138), anti-Rac1 (BD Transduction Laboratories, 610651), and anti β-actin (Sigma-Aldrich, A5441). Membranes were then washed and incubated with either anti-mouse or anti-rabbit antibodies (1:3,000) conjugated to horseradish peroxidase (Amersham Biosciences). Bands were visualized using the ECL Western blot detection system (Amersham Biosciences). Films were scanned, and immunoblot-derived signals were quantified using the Quantity One 1D image analysis software (Bio-Rad).

Subcellular fractionation

COS-1 cells were seeded in 10-cm plates and grown to 90% confluence. Cells were washed twice with PBS and allowed to swell on ice in 1 ml of hypotonic buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, and protease inhibitors (Complete, Roche Molecular Biochemicals) for 10 min. The cell suspension was centrifuged at low speed (2,000 × g for 1 min at 4 °C), and the supernatant was collected as the cytosolic fraction. The pellet (nuclear fraction) was rinsed once in hypotonic lysis buffer, resuspended in 250 μl of nuclear extraction buffer (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, 5 mM β-glycerophosphate, 1 mM DTT, and protease inhibitors), and briefly sonicated. Laemmli sample buffer was added to cytosolic and nuclear extracts, and equal cell equivalents were analyzed by Western blotting as indicated above, with anti GFP for detection of EGFP-tagged β1-Δ7p-chimaerin. Anti-lamin A/C (Novus, 4C4) and anti α-tubulin (Merck, CP06) were used as nuclear and cytosolic markers, respectively.

Active Rac pulldown

Rac-GTP levels in the isolated nuclear fraction were assessed by pulldown assay with a GST fusion protein containing the Rac1 binding domain of PAK1 (GST-PBD), as described previously (19). Briefly, nuclei were isolated from COS-1, A565, and H358 cells as indicated above and lysed in nuclear extraction buffer containing 10 μg of GST-PBD. Lysates were pre-cleared by centrifugation at 14,000 rpm for 10 min at 4 °C and then incubated with GSH-Sepharose beads (GE Healthcare) for 1 h at 4 °C. After extensive washes, samples were boiled in Laemmli sample buffer and separated by electrophoresis. Bound Rac (Rac-GTP) was detected by immunoblotting using an anti-Rac antibody as described above.

Cell cycle analysis

The cell cycle distribution of either exponentially growing COS-1 cells or synchronized cultures was determined by propidium iodide analysis of DNA content. For cell synchronization, cells were grown to 50% confluence in 10-cm dishes for 24 h, followed by incubation with 1.5 mM HU for an additional 16 h. Cells were then released from G1/S block by incubation in fresh growth medium and harvested at the indicated time points. Cells were then washed with PBS, fixed, and incubated with 0.1 mg/ml RNase A (DNase-free, Thermo Scientific) and 40 μg/ml propidium iodide (PI; Sigma) for 30 min. Samples were analyzed on a Gallios flow cytometer (Beckman Coulter), and data were processed using FlowJo V10 software.

Statistical analysis

For statistical analysis, data from at least three independent experiments were used. Data are shown as mean ± S.D. Student’s t test p ≤ 0.05 was considered statistically significant.

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