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Identification of FAM96B as a novel prelamin A binding partner

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ABSTRACT

Prelamin A accumulation causes nuclear abnormalities, impairs nuclear functions, and eventually promotes cellular senescence. However, the underlying mechanism of how prelamin A promotes cellular senescence is still poorly understood. Here we carried out a yeast two-hybrid screen using a human skeletal muscle cDNA library to search for prelamin A binding partners, and identified FAM96B as a prelamin A binding partner. The interaction of FAM96B with prelamin A was confirmed by GST pull-down and co-immunoprecipitation experiments. Furthermore, co-localization experiments by fluorescent confocal microscopy revealed that FAM96B colocalized with prelamin A in HEK-293 cells. Taken together, our data demonstrated the physical interaction between FAM96B and prelamin A, which may provide some clues to the mechanisms of prelamin A in premature aging.

1. Introduction

Hutchinson–Gilford progeria syndrome (HGPS) is a severe childhood disease characterized by accelerated aging [1]. It is caused by mutations in *LMNA*, which encoding A-type lamins (predominantly lamin A and C) [2]. Lamin A is a component of the nuclear lamina that plays a critical role in the structural organization and function of the nucleus. Lamin A is first synthesized as a prelamin A precursor with a C-terminal CaaX motif and undergoes a series of posttranslational modifications including CaaX processing (farnesylation, aaX cleavage and carboxylmethylation), followed by endoproteolytic cleavage by Zmpste24 [3]. Loss of Zmpste24 activity arrests the processing of prelamin A at a stage similar to HGPS, although a unique truncated prelamin A (progerin) is accumulated in HGPS cells [4–6]. Recent studies have found that the similar molecular mechanism responsible for HGPS is active in healthy cells [7,8]. Prelamin A accumulation is not observed in young healthy vessels but is prevalent in vascular smooth muscle cells from aged individuals, which accompanied with nuclear morphology defects. Therefore, the toxic accumulation of prelamin A might be a novel target to ameliorate the effects of age-induced cell dysfunction [8].

To better understand the deleterious effects of prelamin A accumulation, we performed a yeast two-hybrid screen on a human skeletal muscle cDNA library, using the full length of premanin A as a bait to search novel interacting factors. And the family with sequence similarity 96 member B (FAM96B, also known as MIP18) was hunted as a novel binding partner of prelamin A. We further validated their binding affinity using GST-pull down, co-immunoprecipitation and confocal co-localization experiments.

2. Materials and methods

2.1. Yeast two-hybrid screen

A yeast two-hybrid screen was performed according to the Clontech Yeast Protocols Handbook (Clontech, Mountain View, CA). Briefly, cDNA encoding human prelamin A was inserted into the *Eco*R I-*Bam*H I sites of the vector pGBKT7 containing the GAL-4 DNA binding domain (pGBKT7-prelamin A). The pGBKT7-prelamin A bait plasmid was transformed into yeast strain AH109, and then the transformed AH109 mated with yeast Y187 containing pACT2 with human skeletal muscle Matchmaker cDNA library. Positive clones were identified under high stringency conditions, and were defined as clones which exhibited growth on the -Trp, -Leu, -His, -Ade medium, and were positive for galactosidase activity. In order to exclude false positives, the plasmids of positive colonies

Abbreviations: FAM96B, family with sequence similarity 96 member B; HGPS, Hutchinson–Gilford progeria syndrome; CIA, the cytoplasmic Fe–S assembly.

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and pGBKT7-prelamin A were retransformed into yeast strain AH109 and then plated on the same high stringency conditions to test the specificity of interactions. Isolated plasmids were finally sequenced and compared with known sequences in GenBank by BLAST search.

2.2. GST pull down assay

The pGEX4T1-FAM96B vector encoding GST-FAM96B fusion protein was generated by inserting FAM96B cDNA into the *Eco*R I-*Xho* I site of the vector pGEX4T1. The pET32a-prelamin A encoding His-prelamin A fusion protein was generated by inserting prelamin A cDNA into the *Xho* I-*Eco*R I site of the vector pET32a. *E.coli* BL21 (DE3) was transformed with pET32a-prelamin A and pGEX4T1-FAM96B vector or pGEX4T1 empty vector. After induction with 0.4 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (GIBCO BRL, Grand Island, NY) overnight at 25 °C, GST fusion proteins were isolated according to the manufacturer's protocol (NucBuster Protein Extraction Kit, Novagen, Darmstadt, Germany). GST-pull down procedures followed ProFound™ Pull-Down GST Protein: Protein Interaction Kit (Pierce, Rockford, IL, USA).

2.3. Co-immunoprecipitation

HA-FAM96B vector was generated by inserting FAM96B cDNA into the *Eco*R I-*Xho* I site of the vector pCMV-HA. The prelamin A cDNA was inserted into the *Eco*R I-*Xho* I site of pCMV-Myc to attain the myc-prelamin A vector. The pCMV-HA or HA-FAM96B and myc-prelamin A plasmids were cotransfected into human embryonic kidney (HEK-293) cells using LipofectAMINE 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. For immunoprecipitations, cell lysate were pretreated with Protein A agarose and incubated with 10 μl of anti-HA antibody overnight at 4 °C. 100 μl of 50% slurry Protein A agarose was added and the samples were rotated for 2 h at 4 °C. After washing, co-immunoprecipitated proteins were eluted by boiling and analyzed by Western blot.

2.4. Western blot

The extracted proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% defatted milk powder in TBS containing 0.1% Tween 20 for 2 h at room temperature and then probed with the appropriate primary and secondary antibodies. Antibody-antigen complexes were visualized using commercial ECL kit (Pierce, Rockford, IL, USA). The anti-His antibody was obtained from Cell Signaling Technology. Anti-myc, anti-HA and anti-actin antibodies were from Santa Cruz Biotechnology. HRP-conjugated secondary antibody was obtained from Life Technologies.

2.5. Fluorescence confocal microscopy

The pEGFP N1-FAM96B, which contains an enhanced green fluorescent protein (EGFP) expression cassette, was constructed as follows based on plasmid pEGFP N1 (Clontech, Cambridge, UK): The *FAM96B* cDNA was generated by RT-PCR, using primers 5′ CCG CTC GAG ATG GTA GGC GGC GGC GGG GTC 3′ and 5′ G GAA TTC G GGA GCG GGC TGA CAG GCA CT 3′. The amplified DNA fragment was inserted into the *Xho*I and *Eco*RI cloning sites of pEGFP N1. The pDsRed2-N1-prelamin A vector was constructed as follows based on plasmid pDsRed2-N1 (Clontech, Cambridge, UK): The *prelamin A* cDNA was generated by RT-PCR, using primers 5′ ACG CTC GAG ATG GAG ACC CCG TCC CAG CGG C 3′ and 5′ CG GGA TCC GCC ATG AAG AAG CAG TTC TGG GG 3′. The amplified DNA fragment was inserted into the *Xho*I and *Bam*HI cloning sites of pDsRed1-N1. The cloning sequences of all plasmids were confirmed by DNA sequencing.

HEK-293 cells were seeded into 48-well culture plates one day before transfection. When the cells reached ~60% confluency, the pEGFP N1 or pEGFP N1-FAM96B and the pDsRed2-N1 or pDsRed2-N1-prelamin A plasmids were transfected using LipofectAMINE 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The transfected cells were observed the location or co-location of prelamin A and FAM96B by using Leica TCS SP5 confocal microscope operated by Leica confocal software.

3. Results

3.1. FAM96B was identified as a novel prelamin A-binding protein by yeast two-hybrid screen

To identify prelamin A-interacting proteins, we screened a human skeletal muscle cDNA library using the yeast two-hybrid system. With the full length prelamin A as bait, the positive clones, which specifically interacted with BD-prelamin A, but not BD-Null baits under both medium- and high-stringency situation, were sequenced and BLAST searched. FAM96B that had no auto-activation was identified as a potential partner, and then reintroduced into yeast cells to confirm the interaction with pGBKT7-prelamin A bait plasmid (Fig. 1).

3.2. FAM96B interacted with prelamin A in vitro and in vivo

To confirm the interaction between prelamin A and FAM96B, we carried out both *in vitro* GST pull-down (Fig. 2A) and *in vivo* co-immunoprecipitation (Fig. 2B) assays. As shown in Fig. 2A, GST-FAM96B, but not GST was able to interact with prelamin A (Fig. 2A, lane 2 and 3). For further verification of the interaction, we carried out co-immunoprecipitation experiments. Human embryonic kidney HEK-293 cells were transfected with HA-FAM96B or empty vector control and myc-prelamin A, and an immunoprecipitation was performed using anti-HA antibody followed by SDS–PAGE. Interaction with prelamin A was then detected by immunoblotting with anti-myc antibody. As shown in Fig. 2B, myc-prelamin A was present in immunoprecipitations from cell lysates transfected with both proteins (Fig. 2B, lane 3) but not from lysates transfected with myc-prelamin A and HA empty vector (Fig. 2B, lane 4), thus indicating that FAM96B interacts with prelamin A *in vivo*.

3.3. FAM96B colocalized with prelamin A within cells

Once the physical interaction between FAM96B and prelamin A had been confirmed, we investigated whether the two proteins colocalized within the cell. We first analyzed the subcellular localization of FAM96B and prelamin A by transfected with the GFP-

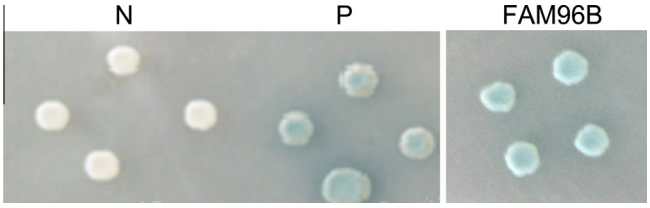

Fig. 1. Analysis of the interaction between FAM96B and prelamin A using the yeast two-hybrid assay. N, AH109 transformed with pGBKT7-prelamin A was used as negative control. P, AH109 transformed with pGADT7-RecT and pGBKT7-53 was used as positive control according to the Clontech Yeast Protocol. The blue signal indicates activation of the reporter genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

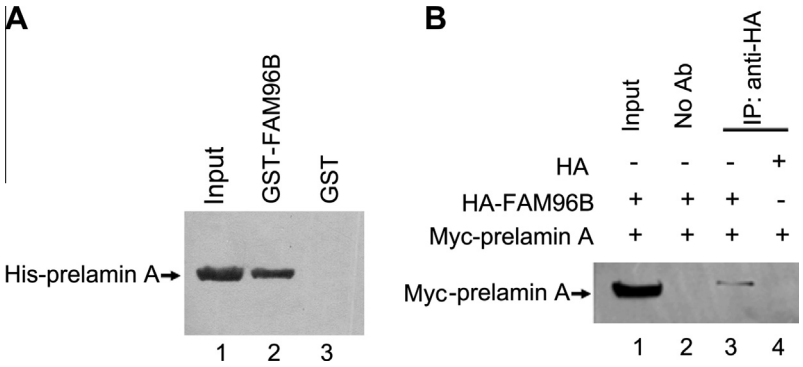

Fig. 2. *In vitro* and *in vivo* confirmation of the FAM96B-prelamin A interaction. (A) Confirmation of FAM96B and prelamin A interaction by GST-pull down. GST-bound proteins were captured by glutathione Sepharose and separated by SDS–PAGE. His-prelamin A was identified by immunoblotting with anti-His antibody. (B) Co-immunoprecipitation of FAM96B and prelamin A. HEK-293 cells were co-transfected with pCMV-HA or HA-FAM96B and Myc-prelamin A and then subjected to immunoprecipitation with anti-HA antibody, followed by immunoblotting with anti-Myc antibody.

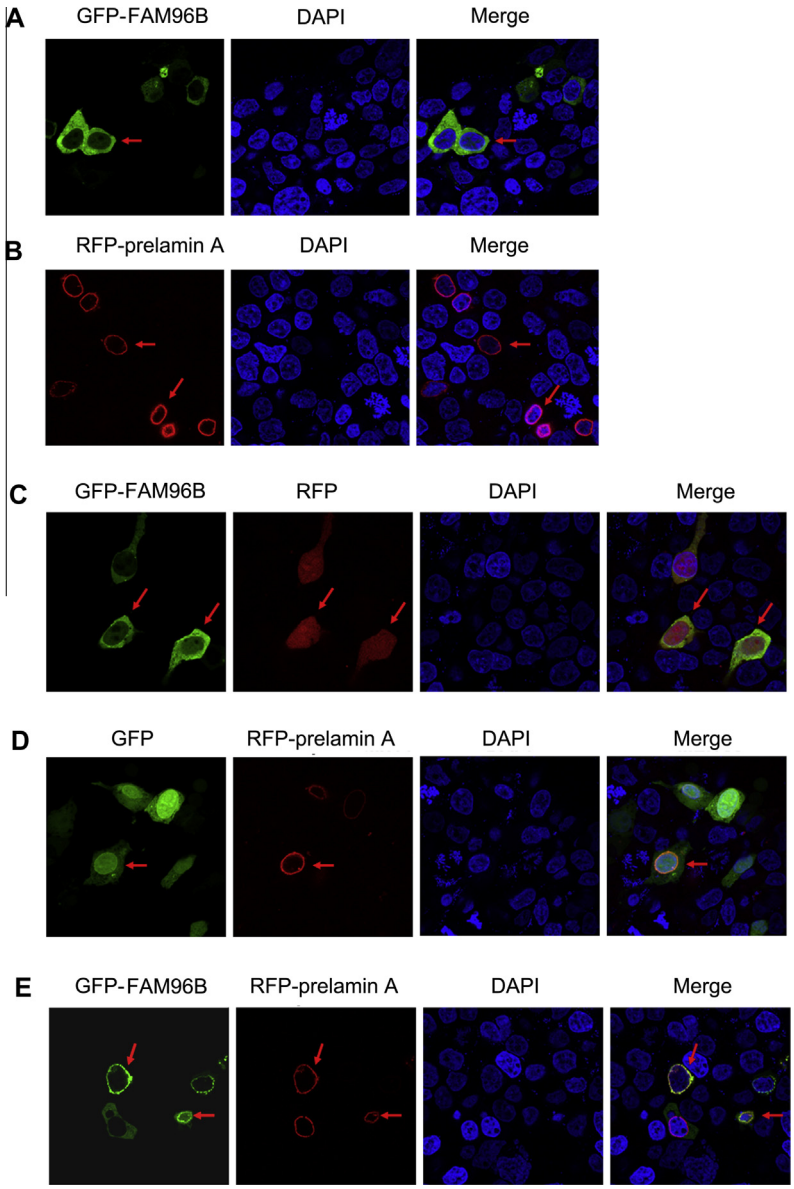

Fig. 3. Co-localization of FAM96B and prelamin A in HEK-293 cells. HEK-293 cells were transfected with GFP-FAM96B (A) or RFP-prelamin A (B) or co-transfected with GFP-FAM96B and pDsRed1-N1 (C) or co-transfected with pEGFP-N1 and RFP-prelamin A (D) or co-transfected with GFP-FAM96B and RFP-prelamin A (E). 24 h after transfection, the cells were stained with DAPI and examined by confocal scanning laser microscopy. Arrowheads indicate the subcellular localization of GFP and (or) RFP-tagged proteins.

tagged FAM96B or RFP-tagged prelamin A alone. As shown in Fig. 3A, FAM96B was detected mainly in the cytoplasm. In contrast, prelamin A was detected predominantly in the nuclear rim (Fig. 3B), as previously reported [4,9]. In addition, co-transfection of GFP-tagged FAM96B and RFP-mock plasmids (Fig. 3C) or GFP-mock and the RFP-tagged prelamin A plasmids (Fig. 3D) in HEK-293 cells did not change the subcellular localization of either GFP-FAM96B or RFP-prelamin A. However, co-transfection of the GFP-tagged FAM96B and the RFP-tagged prelamin A markedly changed the subcellular localization of FAM96B from cytoplasm to nuclear envelope, where it exhibited excellent co-localization with prelamin A (Fig. 3E). These results established a direct connection between FAM96B and prelamins A, suggesting that physical interaction between the two proteins might give rise to the retention of FAM96B at the nuclear envelope.

4. Discussion

Growing evidences have suggested that prelamin A accumulation could induce DNA damage, leading to genomic instability, and premature senescence [5,6,8]. However, the underlying mechanism of how prelamin A promotes cellular senescence is still poorly understood. Previous reports showed that prelamin A interacted with several proteins, including Narf [10], Emerin [11], SREBP1 [12] and SP1 [13]. In the present study, we screened prelamin A binding proteins by the yeast two-hybrid system, and identified FAM96B as a novel binding protein of prelamin A in addition to NARF. The interaction of FAM96B with prelamin A was further confirmed by GST pull-down assay and co-immunoprecipitation. Co-localization experiments by fluorescent confocal microscopy revealed that prelamin A accumulation could stimulated retention of FAM96B at the nuclear envelope, which indicated a functional importance of the interaction. Together, our results unveiled that FAM96B physically interacted with prelamin A, and the interaction may provide some clues to the mechanisms of prelamin A in premature aging.

FAM96B has been identified as an interaction partner of E2-2, which promoted decrease of E2-2 protein to rescue E2-2-mediated repression of VEGFR2 promoter activity[14]. Therefore, FAM96B concomitantly enhanced endothelial cell migration, proliferation, and tube formation [14]. In addition, FAM96B was also shown to be part of the MMXD complex (MMS19-FAM96B-XPD), which played a crucial role in mitotic spindle formation and chromosome segregation [15]. Knockdown of FAM96B increased heterotype nucleus accumulation [15]. Recently, FAM96B was implicated in the cytoplasmic Fe–S assembly (CIA) machinery as part of a complex that included CIAO1, MMS19, and NARFL, three known components of the CIA system [16–18]. Furthermore, FAM96B was required for cytosolic Fe–S cluster assembly of several Fe–S proteins involved in DNA metabolism, and played an important role in DNA repair and genomic instability [16,17].

Subcellular localization of proteins was tightly linked to its biological function or stability [19]. In this study, transient expression of GFP-tagged FAM96B in human embryonic kidney HEK-293 cells clearly showed that exogenous FAM96B localized predominantly in the cytoplasm. However, overexpression of RFP-tagged prelamin A, which localized mainly at the nuclear envelope [4,9], caused a large fraction of GFP-FAM96B to redistribute from the cytoplasm to the nuclear periphery, convinced the association between FAM96B and prelamin A in HEK-293 cells. To explain the change of intranuclear localization, we speculated that the accumulation of prelamin A mistakenly recruited the FAM96B from cytoplasm to the nuclear envelope, and, by restricting its access to other cellular locations where it functions, resulted in genomic instability and cellular senescence.

For the deep insights on the binding site of FAM96B in prelamin A, we noticed that *LMNA* mutations in HGPS mainly concentrated on the C-terminus [20], indicating proteins specifically interacting with the C-terminus of prelamin A might affect progeroid syndromes. Therefore, to probe into the localization of FAM96B binding sites, we performed a yeast two-hybrid screen on a human skeletal muscle cDNA library using the C-terminus of prelamin A (amino acids 389–664) as a bait. We also found that the association of the C-terminal tail of prelamin A with FAM96B (data not shown). These data revealed that FAM96B might interact with the C-terminal tail of prelamin A, suggesting possible roles of FAM96B on premature aging.

In summary, our study provided the first evidence that FAM96B physically interacted with prelamin A, suggesting possible roles of FAM96B on cellular senescence. Further studies are necessary to clarify the underlying mechanism for the prelamin A-Fam96B interaction with regard to premature aging.

Acknowledgments

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