ORIGINAL ARTICLE

p60-katanin: a novel interacting partner for p53

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Abstract

Katanin, one of the best-characterized microtubule (MT) severing proteins, is composed of two subunits: catalytic p60-katanin, and regulatory p80-katanin. p60-katanin triggers MT reorganization by severing them. MT reorganization is essential for both mitotic cells and post-mitotic neurons in numerous vital processes such as intracellular transport, mitosis, cellular differentiation and apoptosis. Due to the deleterious effect of continuous severing for cells, p60-katanin requires a strategic regulation. However, there are only a few known regulators of p60-katanin. p53 functions in similar cellular processes as katanin such as cell cycle, differentiation, and apoptosis depending on its interacting partners. Considering this similarity, in this study we investigated p53 as a potential regulatory candidate of p60-katanin, and examined their interaction. Co-immunoprecipitation analyses revealed that p60-katanin interacts with p53. We were able to locate a potential interaction site for the two proteins by deleting different candidate regions We showed for the first time that p53 and p60-katanin interact. This interaction appears to occur via p53's DNA binding domain and p60-katanin's C-terminal. This study will pave the way for future studies regarding the functional outcomes of this interaction which is vital for understanding the regulation of cellular events such as cell cycle, differentiation, and apoptosis in disease and in health.

Introduction

Katanin is a member of the ATPase family, a microtubule (MT) severing enzyme [1] which is named after the sword of the Japanese Samurai, Katana. It consists of two subunits: p60-katanin encoded by the *KATNA1* gene and p80-katanin encoded by the *KATNB1* gene. p60-katanin is responsible for enzymatic activity, whereas p80-katanin is involved in the regulation and localization of p60-katanin

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[2–4]. p60-katanin triggers MT reorganization by severing them. The reorganization of MTs is functional for mitotic cells especially in the cell division, while in post-mitotic cells such as neurons, the reorganization of MTs is vital for morphological and functional differentiation of neurons by the formation of axons and dendrites.

In proliferating cells, p60-katanin promotes mitosis by severing the MTs in the mitotic spindle poles and creating a large number of minus MT ends, thereby ensuring gamma-tubulin accumulation in mitotic centrosomes which forms the nucleation center for MTs [5]. On the other hand, p60-katanin severs and disassembles MTs at spindle poles to shorten the metaphase spindles [6]. However, in post-mitotic neurons, p60-katanin directs all its power to severe MTs in order to form neuronal extensions such as axons, a process called "neurite growth" which is essential for neuronal network formation [7, 8]. Although MTs are the main cytoskeletal component of the axons, they are too long to be transported into axonal extensions during neurite growth [9]. Thus, p60-katanin severs long MTs into shorter fragments to be easily transported by motor proteins to the axons [10]. Based on all this information, it is evident that p60-katanin is a critical protein

that plays a role in the most vital activities of both mitotic and post-mitotic cells.

Unless its severing activity is controlled, p60-katanin would continuously severe MTs which is undesirable for the cells. Thus, it is evident that katanin requires a strategic regulation within the cell. Despite the fact that MT severing mechanism of p60-katanin has been studied extensively for years, little is known about its regulation. There are almost no known regulators of katanin. Therefore, revealing the regulation partners of p60-katanin is of primary importance for understanding the functional regulation of this protein, the MT severing dynamics and also the reciprocal effects of these interactions. In this regard, analyzing the interactions of p60-katanin with its partners will provide important insights about the regulatory mechanism of essential cellular events such as cell cycle and cellular differentiation.

Similar to p60-katanin, tumor suppressor p53 plays an essential role in the pendulum between proliferation, apoptosis and differentiation by binding to different proteins within the cell. p53, also known as a master guardian that controls proliferation in dividing cells, has been shown to be associated with differentiation by affecting neuronal process lengths in non-dividing neurons [11–14].

Furthermore, our previous study that was conducted with primary neurons clearly showed that activating cell cycle by triggering Protein Kinase C caused retraction of neuronal processes with a concomitant increase in p60-katanin levels. Based on this observation, p53 level was then examined and it was indicated that p53 protein level increased by 1.7-fold [15]. This concurrent increase of p53 and p60-katanin proteins suggested that p53 and p60-katanin could affect each other.

Considering the critical role of p60-katanin in MT regulation, especially in mitotic spindle formation for mitotic cells and in the formation of functional structures of neuronal morphology for postmitotic neurons, it is most likely to interact with p53, which has an incontrovertible role in the same processes. To date, p53 has been reported to function in the regulation of proteins responsible for neuronal differentiation such as Koronin1b, Rab13, GAP 43 [14] and it supports the idea that it may interact with another neuronal differentiation regulator, p60-katanin.

Regarding the fact that both p60-katanin and p53's critical roles in proliferation and differentiation, we aimed to answer the question: "Is it possible for them to interact in the cell?". For this purpose, first, we determined p53–p60-katanin interaction, then identified the possible regions of this interaction. Results of the study showed for the first time that p53 and p60-katanin do interact. The interaction appears to occur via p53's DNA binding domain and C-terminal of p60-katanin.

Materials and methods

Cell culture

Rat RFL-6 cells were a kind gift from Prof. Arzu Karabay (Istanbul Technical University) and were maintained in medium containing F12K (Lonza, Basel, Switzerland), 20% fetal bovine serum (heat inactivated, E.U. 10500064, Thermo Fisher, Gibco), Non-Essential Amino Acids (BE13-114E, Lonza) and 2 mM L-Glutamine (25030081, Thermo Fisher, Gibco). The cells were cultivated in a 5% CO2 incubator at 37 °C and plated at a density of 5×10^5 cells/well in a 6-well plate for Immunoprecipitation and Western blotting experiments.

Cell extraction

Total protein extraction was performed by using Mammalian Cell Extraction Kit (K269-500, BioVision, San Francisco Bay, San Francisco, USA) and specified as Whole Cell Lysate (WCL) through the study. The protein concentration was measured using Bicinchoninic Acid protein assay kit (23235, Pierce, Waltham, MA, USA).

Deletion constructs

All deletion constructs were obtained by cloning different parts of both Wild Type (WT) p53 (AB082923) and WT p60-katanin (NM_007044) (Fig. 3). p60-katanin and p53-deletion constructs were prepared by sub-cloning different parts of WT genes into pcDNA3.1/myc-His and 3XFLAG-CMV™-10 vectors respectively. Prepared constructs were: Transcription Activation Domain (TAD-p53, p53-b), DNA Binding Domain (DBD-p53, p53-c) and Oligomerization-Regulatory Domain (OD-RD-p53, p53-d) constructs for p53 (Fig. 3a); C-terminal containing construct (C-term-p60-katanin, p60-katanin-b) and del201-268 construct (del201-268-p60-katanin, p60-katanin-c) for p60-katanin (Fig. 3b).

Transient transfection

Cells were seeded in 6 well-plates at a density of 5×10^5 and were cultured overnight in a humidified incubator (5% CO₂, 37 °C). Following day, after confluence of the cells reached 70–90%, chemical transfection was performed by using liposome-based transfection reagent, Lipofectamine 3000 (L3000-001, Thermo Fisher Scientific, Waltham, MA, USA). In co-immunoprecipitation (Co-IP) Co-IP experiments, for total 2 μg of DNA, different combinations of 1 μg of p53 and 1 μg of p60-katanin deletion constructs were mixed with P3000

reagent in Opti-MEM Medium (31985062, Thermo Fisher, Gibco). These DNAs were then mixed with Lipofectamine 3000 reagent which was diluted in Opti-MEM Medium. The transfection mixture was incubated in room temperature for 15 min and applied onto the cells drop-by-drop. Cells were cultivated for 48 h in 37 °C, 5% CO_2 incubator. Finally, total protein extraction was performed by using BioVision Mammalian Cell Extraction Kit. A certain amount of total protein was aliquoted as input and the rest of the lysate was used in further Co-IP experiments.

Co-immunoprecipitation & Western blotting

WCL was mixed with primary mouse anti-p53 antibody (1:500, 2524, Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. Next day, antibody–lysate mixture was mixed with Protein G magnetic beads in 4 °C rotator for 1 h. Unbound proteins were washed away for 3 times with PBS and bound proteins were eluted using 50 mM Glycine buffer (pH 2.8). For non-specific IgG and no antibody (beads only) reactions as the negative controls; the same amount of lysate was mixed with either 3 μg normal mouse IgG or the lysate was used without antibody, respectively. Positive control for Co-IP was the total protein lysates aliquoted as input. Eluted proteins, IgG and input samples were separated by 4–12% Bis–Tris SDS-PAGE and transferred onto a nitrocellulose membrane. Possible interaction of immunoprecipitated constructs was detected by immunoblotting via rabbit anti-p60-katanin antibody (1:1000, HPA036207, Atlas Antibodies, Bromma, Sweden), mouse anti-p53 antibody (1:500, 2524, Cell Signaling Technology, Danvers, MA, USA). HRP-conjugated antibodies (goat anti-mouse IgG-HRP: sc-2031; goat anti-rabbit IgG-HRP: sc-2004) were used as the secondary antibody (1:5000, Santa Cruz Biotech., Dallas, TX, USA). Visualization of the protein bands was performed using colorimetric Amplified Opti-4CN Detection Kit (1708238, Bio-Rad, Hercules, CA, USA).

To reveal the domains that have a role in the interaction, WCL was obtained from the cells that were transfected with deletion constructs. Firstly, 4 μg of mouse monoclonal anti-Octa (FLAG) Antibody (sc-166355, Santa Cruz Biotech., Dallas, TX, USA) was mixed with 500 ng of lysate and incubated overnight at 4 °C. The Co-IP analysis was performed as explained previously in detail. Possible interaction of immunoprecipitated constructs was detected by immunoblotting via rabbit monoclonal anti-His primary antibody (1:1000, 2365S, Cell Signaling Technology, Danvers, MA, USA).
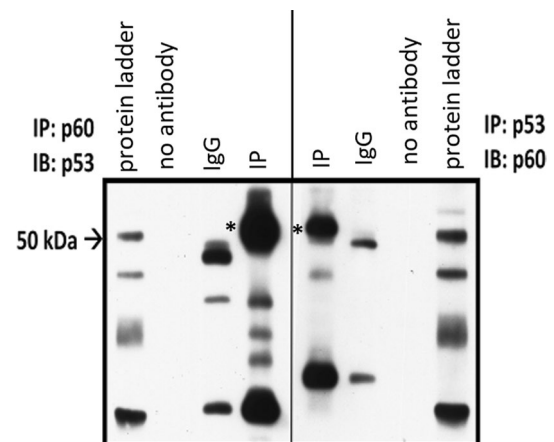
Results

p60-katanin interacts with p53

To examine the possible interaction between p60-katanin and p53, we performed co-immunoprecipitation for endogenous p60-katanin and p53 (Fig. 1 and Supplementary Fig. S1). For this purpose, cell lysates were precipitated with either anti-p60-katanin or anti-p53 katanin and immunoblotted with anti-p53 and anti-p60-katanin, respectively. As Co-IP data indicates (Fig. 1, IP *) that p60-katanin and p53 interact.

Interaction is provided through C-terminal of p60-katanin and DNA binding domain of p53

To locate the regions that have a role in this interaction, different FLAG- or His-tagged deletion constructs were prepared for both p53 and p60-katanin, respectively (Fig. 2). Besides WT p53 (WT-p53, p53-a), we prepared Transcription Activation Domain (TAD-p53, p53-b), DNA Binding Domain (DBD-p53, p53-c) and Oligomerization-Regulatory Domain (OD-RD-p53, p53-d) constructs for p53 (Fig. 2a). Similarly, besides WT p60-katanin (WT-p60-katanin, p60-katanin-a), C-terminal containing construct (C-term-p60-katanin, p60-katanin-b) and del201-268 construct (del201-268-p60-katanin, p60-katanin-c) for p60-katanin were prepared (Fig. 2b).

Different combinations of deletion constructs were transferred into the cells by chemical transfection. As
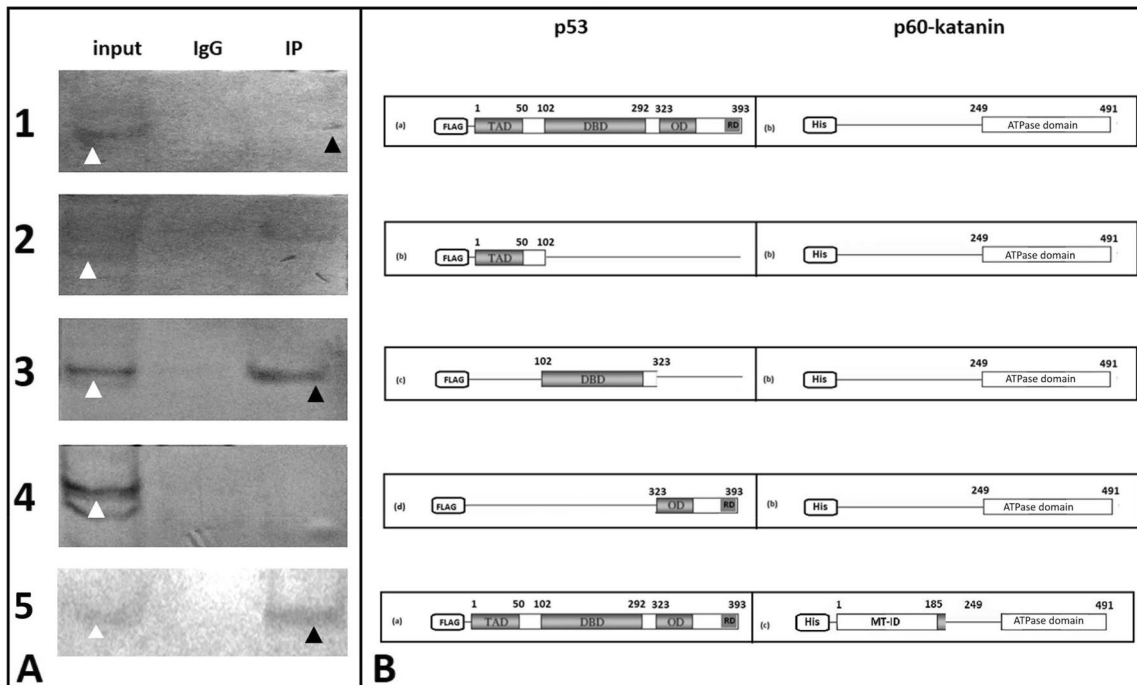

Fig. 1 Western blotting result for co-immunoprecipitation of endogenous p60-katanin and p53. WCL were immunoprecipitated (IP) with either anti-p60-katanin or anti-p53 antibody and immunoblotted (IB) with either anti-p53 or anti-p60-katanin antibody, respectively. Asterisk (*) indicates an interaction between p60-katanin and p53. In IgG negative control, there is non-specific antibody during IB, no antibody control includes only beads during IB
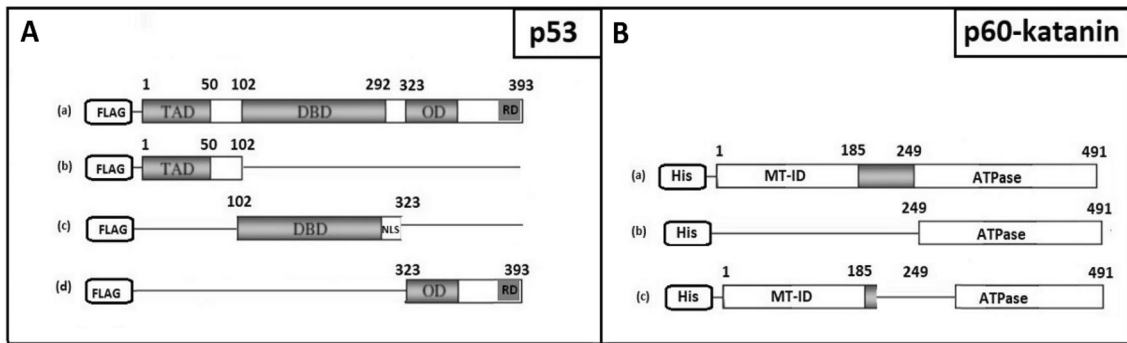
Fig. 2 Schematic illustration of deletion constructs. Represents the deletion constructs for either p53 (**a**) or p60-katanin (**b**)


Fig. 3 Western blotting image of co-immunoprecipitation analysis. The left part of the figure shows Co-IP analyses results (**a**) and on the right part there is an illustration of deletion constructs used in Co-IP experiments (**b**). Input (total protein extraction) is positive control and IgG indicates negative control performed with non-specific IgG.

Lines pointed with either white (in input) or black (in IP) arrowhead show the desired protein signal. Immunoprecipitation was done with anti-FLAG antibody to precipitate FLAG-tagged construct [either WT-p53 (1, 5), TAD-p53 (2), DBD-p53 (3) or OD-RD-p53 (4)]

illustrated in Fig. 3, first transfection was performed with WT-p53 and C-term-p60-katanin. As Co-IP data indicated (Fig. 3a, 1) analysis was positive in terms of interaction since the signal was in line with input. The C-term-p60-katanin construct was further tested for the interaction with either TAD-p53 (Fig. 3a, 2), DBD-p53 (Fig. 3a, 3) or OD-RD-p53 (Fig. 3a, 4). Last combination that was tested

was for WT-p53 and del201-268-p60-katanin (Fig. 3a, 5). Analyzing whole Co-IP data clearly indicated that C-term-p60-katanin construct interacts only with DBD-p53. Hereby, interaction between p53 and p60-katanin appears to be through the C-terminal of p60-katanin and DNA Binding Domain of p53 (Fig. 4).
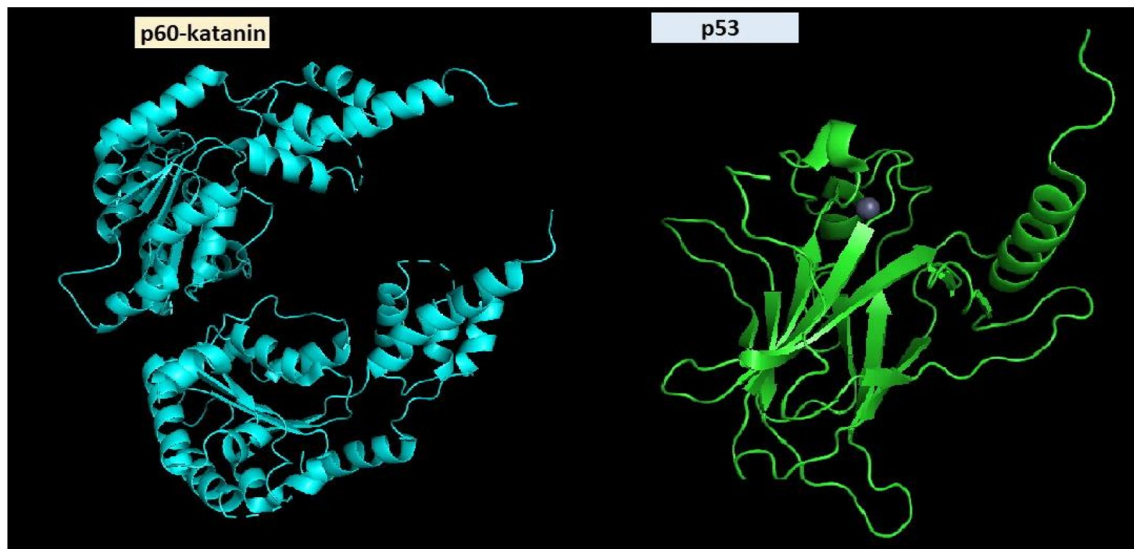
Fig. 4 Schematic illustration of interacting domains. Domain illustrations are generated via PyMOL and data were taken from https://www.rcsb.org/ for p60-katanin (5ZQL, crystal structure of human katanin AAA ATPase domain) and p53 (2FEJ, solution structure of human p53 DNA binding domain)

Discussion

MTs, being essential elements of the cell, are involved in essential functions such as intracellular cargo transport, cell motility, and cell division as well as specialized functions such as formation, growth and maintenance of axons and dendrites in neurons [1, 3]. Besides their inherent dynamic instability, MT severing proteins have also inevitable function in MT reconfiguration. Katanin is one of the best-characterized MT severing proteins and is composed of two subunits, regulatory subunit p80-katanin, and catalytic subunit p60-katanin [16] of which regulatory partners are still little-known. Similar to p60-katanin, p53 is also important in controlling different cellular mechanisms such as cell cycle arrest, senescence, differentiation and apoptosis by regulating the expression of regulatory proteins [11–14]. Since both p60-katanin and p53 have critical roles in proliferation and differentiation, a reasonable question to ask is: "Is it possible for p60-katanin and p53 to be partners in the cell?".

To answer this critical question, the first aim of this study was to determine whether p53 and p60-katanin interact in the cell. Our findings revealed that p53 and p60-katanin interact in the cell (Fig. 1). Considering that katanin requires a strict regulation due to its critical MT severing action and p60-katanin and p53 have roles in similar essential cellular events such as proliferation, apoptosis and differentiation, it seems reasonable for these proteins to interact in the cell.

Besides identifying the interaction between p53 and p60-katanin, we also identified the possible regions of this interaction by using different FLAG- or His-tagged deletion and wild type constructs for p53 and p60-katanin.

Our results indicated that p53 interacts with p60-katanin through p60-katanin's C-terminal domain—which also includes ATPase domain (Fig. 4). Through this interaction, it is most likely that p53 may affect the MT severing activity of the p60-katanin which is directed by its C-terminal domain, and thereby indirectly the MT dynamics, depending on intracellular conditions.

The analysis of the interacting regions also revealed that the communication of p60-katanin with p53 takes place via the DNA binding domain of p53. This result indicates that p60-katanin may affect DNA binding function of p53. As with most transcription factors, p53 has various protein–protein interaction sites which are crucial for the fine-tuning of its activity. In concordance with our results, there is a number of protein partners that interact p53 via its DNA-binding domain. Among them, Hypoxia inducible factor-1 alpha (HIF-1a) [17, 18], Heat shock protein 90 (Hsp90) [19], Rad51 [20, 21], Bcl-XL/Bak [22, 23] are the most remarkable. Looking at all these studies, it is evident that these proteins interact with p53 to regulate important cellular activities such as protein homeostasis, DNA damage repair, cell proliferation, apoptosis and senescence. However, DNA-binding site of p53 is highly charged to be able to electrostatically interact with DNA. Thus, interaction of all these proteins with the DNA-binding site of p53 is strongly dependent on ionic strength as a requirement of electrostatic interaction [18]. For this reason, conditional experiments should be conducted in the future to examine the details of their interaction under different ionic strengths and different pH conditions.

Considering all these data, it is reasonable to conclude that the interaction between p60-katanin and p53 can have functional results for both proteins, in other words, this interaction is a mutual interaction. The results of this study show that both proteins may have a regulatory effect on each other's activity in the cell. However, to be able to suggest such a reciprocal regulatory effect, mechanistic studies should be conducted. Identifying their localization of interaction may provide valuable insights about their regulatory effect on each other. Furthermore, revealing their mode of interaction, whether direct or via MTs is also crucial to deepen our understanding of functional outcomes of their interaction.

Although there is no reported mutation in p53 of RFL-6 cell line in the literature, and also RFL-6 cell line is not found on the ATTC's "Validated p53 Hotspot Mutation Cell Line List" [24], it is crucial for our study to have an intrinsic wild type p53 in the cell because it may affect the significance of the interpretations about the functional outcomes of this cytoplasmic interaction. Thus, further experiments should be conducted in order to make sure whether p53 of RFL-6 cell line is wild type or mutated.

In summary, we showed for the first time that p53 and p60-katanin do interact and this interaction appears to occur via p53's DNA binding domain and p60-katanin's C-terminal domain (Fig. 4). Further elucidating the functional outcomes of this interaction is vital for better understanding of the regulatory mechanism of essential cellular events such as cell cycle, differentiation, and apoptosis in disease and in health.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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