The p53 target protein Wig-1 binds hnRNP A2/B1 and RNA Helicase A via RNA

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Abstract The p53-induced Wig-1 gene encodes a double stranded RNA-binding zinc finger protein. We generated Saos-2 osteosarcoma cells expressing tetracycline-inducible Flag-tagged human Wig-1. Induction of Wig-1 expression by doxycycline inhibited cell growth in a long-term assay but did not cause any changes in cell cycle distribution nor increased fraction of apoptotic cells. Using co-immunoprecipitation and mass spectrometry, we identified two Wig-1-binding proteins, hnRNP A2/B1 and RNA Helicase A, both of which are involved in RNA processing. The binding was dependent on the presence of RNA. Our results establish a link between the p53 tumor suppressor and RNA processing via hnRNPA2/B1 and RNA Helicase A.

Structured summary:
MINT-6542926, MINT-6542899:
WIG1 (uniprotkb:Q9HA38) *physically interacts* (MI:0218) *with hnRNP A2/B1* (uniprotkb:P22626) *by anti bait coimmunoprecipitation* (MI:0006)
MINT-6542945:
RHA (uniprotkb:Q08211) *physically interacts* (MI:0218) *with hnRNP A2/B1* (uniprotkb:P22626) *by anti bait coimmunoprecipitation* (MI:0006)
MINT-6542918, MINT-6542891:
WIG1 (uniprotkb:Q9HA38) *physically interacts* (MI:0218) *with RHA* (uniprotkb:Q08211) *by anti bait coimmunoprecipitation* (MI:0006)
MINT-6542867:
WIG1 (uniprotkb:Q9HA38) *physically interacts* (MI:0218) *with RHA* (uniprotkb:Q08211) *by anti tag coimmunoprecipitation* (MI:0007)
MINT-6542879:
WIG1 (uniprotkb:Q9HA38) *physically interacts* (MI:0218) *with hnRNP A2/B1* (uniprotkb:P22626) *by anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Wig-1 is a target of the p53 tumor suppressor [1]. p53 is a transcription factor that triggers cell cycle arrest, apoptosis and/or senescence in response to cellular stress [2]. This raises the question whether Wig-1 is a downstream effector in one or more of these processes. Wig-1 has three Cys_2His_2-type zinc finger domains that are highly conserved from fish to man [1,3]. The structural features of the Wig-1 zinc fingers are shared with the RNA-binding proteins dsRBP-ZFa and JAZ. We found that Wig-1 binds dsRNA with high affinity and that the first and second zinc fingers are necessary for binding to dsRNA in living cells [4,5]. Exogenous Wig-1 causes a modest growth inhibition in a colony formation assay [1].

hnRNPs participate in transcriptional regulation, maintenance of telomere length, alternative pre-mRNA splicing and pre-mRNA 3′ end processing in the nucleus. In the cytoplasm, hnRNPs can regulate mRNA localization, translation and turnover [6]. hnRNP A2 (36 kDa) and B1 (38 kDa) are isoforms derived from the same gene and differ by only 12 amino acids, due to the presence of exon 2 in the B1 transcript [7]. Targeting of hnRNP A2/B1 promotes cell death in transformed cells but not in primary cells [8], and suppression of hnRNP A2 causes a non-apoptotic inhibition of cell proliferation [9].

RNA Helicase A (RHA, Nuclear DNA Helicase II (NDHII)) is a 142 kDa nuclear helicase that is involved in processes such as transcriptional regulation and RNA processing [10]. Following transcriptional inhibition RHA is translocated to the cytoplasm where it binds mRNAs and pre-mRNAs [11]. RHA is necessary for translation of selected mRNAs by recognising a 5′ post-transcriptional control element [12].

Here we have generated Saos-2 cells carrying Tet-inducible Wig-1 with the aim of examining the function of Wig-1 and identifying potential partner proteins. We show that induction of Wig-1 in these cells inhibits cell growth without any changes in cell cycle distribution or increased apoptosis. Moreover, we identified hnRNP A2/B1 and RHA as Wig-1-binding proteins.

2. Materials and methods

2.1. Cells and cell culture
Saos-2 osteosarcoma cells expressing tetracycline-regulated Flag-tagged Wig-1 have been described [4]. Cells were grown in Iscove's modified Dulbecco's medium with 10% fetal bovine serum with

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reduced tetracycline (Clontech, USA) or normal fetal bovine serum (Sigma–Aldrich, Germany), 2 mM L-glutamine and 40 µg/ml gentamicin (Invitrogen, BV, Netherlands). Wig-1 expression was induced by adding 1 µg/ml doxycycline to the culture media for 48 h unless otherwise stated.

2.2. Immunofluorescence staining

Saos-2 Tet-on and Saos-2 Tet-on-Wig-1 cells were plated on cover slips in 6-well plates, induced with doxycycline and stained with anti-Flag M2 antibody (Stratagen, CA) [1]. Endogenous Wig-1 was stained with Wig-1 antibody (Proteintech Group Inc., USA) in Saos-2 cells.

2.3. WST-1 proliferation assay

Cells cultured on 96-well plates in triplicates were analysed after 96 h or 5 days for growth suppression using the WST-1 assay (Roche Diagnostics, Sweden). Absorbance of samples was measured at 490 nm.

2.4. FACS analysis

Cells were cultured in the absence or presence of doxycycline, fixed in 70% ethanol for 18 h at 4 °C, pelleted and incubated in PBS with propidium iodide and RNase A for 30 min at 37 °C. DNA content was analysed by flow cytometry in a BD FACS cytometer (BD Biosciences).

2.5. Immunoprecipitation and Western blotting

Cells were lysed in VS buffer (50 mM NaCl, 0,5% NP40, 1% protease inhibitor cocktail (Sigma–Aldrich), 1 mM Na_3VO_4 and 10 mM Tris pH 7.5) and rotated at 4 °C for 15 min. Lysates were centrifuged at 12000 rpm for 10 min at 4 °C, the supernatant was treated with 200 µg/ml RNase A at 30 °C for 20 min, precleared overnight with protein A agarose (Amersham Pharmacia Biotech, Sweden or Invitrogen), and incubated with antibody for 6 h. Thirty microliters of protein A Sepharose (Sigma–Aldrich) or 15 µl protein A agarose (Invitrogen) were added and incubated overnight. Proteins were separated on 10% SDS page gels, stained by Silverquest (Invitrogen), Coomassie or blotted onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech) for Western blotting. Antibodies for immunoprecipitation and Western were Flag M2 antibody (Stratagen), anti-RHA (from Chee-Gun Lee, UMDNJ-New Jersey Medical School, NJ [13]), anti-hnRNP A2/B1 (Santa Cruz, USA) 1:250, anti-Wig-1 (see [4]) or anti-β-actin (Sigma–Aldrich). Ten microliters of protein G Dynabeads (Dynal, Norway) was used for immunoprecipitations of endogenous proteins.

2.6. Mass spectrometry

Protein spots were excised and digested using a MassPrep robotic protein handling system (Waters), digested with trypsin (modified; Promega, USA) and applied to plates for MALDI mass spectrometry in a Voyager DE-PRO instrument (PE Applied Biosystems, USA) in positive ion reflection mode. The Protein Prospector web site (http://prospector.ucsf.edu) was used for protein identification.

2.7. siRNA

siRNA oligos: Hs_WIG1_1_HP, Hs_WIG1_2_HP, Hs_HNRNPA 2B1_5_HP validated and AllStars Negative Control siRNA were used. Transfections were carried out using HiPerFect (Qiagen, Germany) according to manufacturer's protocols.

3. Results

3.1. Saos-2 cells expressing Tet-regulated Wig-1

We generated Saos-2 cells carrying Tet-inducible Flag-tagged Wig-1. Western blotting demonstrated that expression levels of Wig-1 correlated with the doxycycline concentration in the medium (Fig. 1A). Immunofluorescence staining showed expression of Wig-1 in the nucleus of doxycycline-treated Saos-2 Tet-on-Wig-1 cells (Fig. 1B), consistent with the nuclear localization of endogenous wild-type Wig-1 (Fig. 1C). We also generated Saos-2 cells carrying Tet-regulated Wig-1 zinc finger
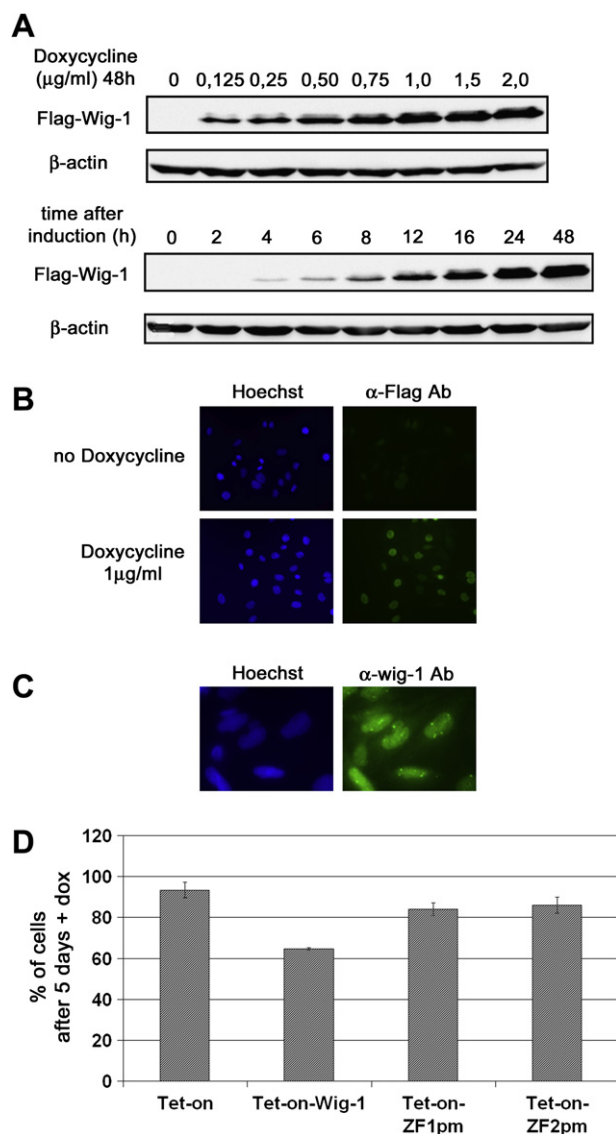

Fig. 1. Saos-2 cells expressing Tet-regulated Wig-1. (A) Western blot analysis of Saos-2 Tet-on-Wig-1 cells cultured with the indicated concentrations of doxycycline (upper panel), or harvested at the indicated time points after induction with doxycycline (lower panel). (B) Immunofluorescence staining of Saos-2 Tet-on-Wig-1 cells with Flag antibody before and after induction with doxycycline. (C) Immunofluorescence staining of Saos-2 cells with Wig-1 antibody. (D) Saos-2 Tet-on, Saos-2 Tet-on-Wig-1, Saos-2 Tet-on-ZF1pm and Saos-2 Tet-on-ZF2pm cells were plated on 96-well plates in triplicates in the presence or absence of 1 µg/ml doxycycline. Cells were analysed after 5 days for growth suppression using the WST-1 cell proliferation reagent. The graph shows percentage of cells cultured with doxycycline compared to cells cultured without.

1 (ZF1) and zinc finger 2 (ZF2) point mutants, both of which fail to bind dsRNA in living cells [4]. Cells expressing the ZF1 and ZF2 point mutants, referred to as Saos-2 Tet-on-ZF1pm and Saos-2 Tet-on-ZF2pm, showed similar inducible expression as the wild-type Wig-1-carrying cells (not shown).

3.2. Wig-1 inhibits tumor cell growth without any effect on cell cycle distribution

We previously showed that Wig-1 inhibits growth in a colony formation assay [1,4]. To examine the short and long-term effects of Wig-1 on cell growth and/or survival in further detail,

we performed WST-1 assays for 5 days in Saos-2 Tet-on cells (carrying only the tet-operator), and Saos-2 Tet-on-Wig-1 cells with or without doxycycline. The Wig-1-expressing cultures contained significantly fewer cells as compared to the same cells grown without doxycycline. We did not observe any significant growth inhibitory effect of the ZF1pm and ZF2pm point mutants (Fig. 1D), in agreement with our previous study [4]. Saos-2 Tet-on and Saos-2 Tet-on-Wig-1 cells cultured in the presence or absence of doxycycline for up to 14 days displayed no changes in cell cycle distribution or increase in the fraction of cells with sub-G1 DNA content as judged by propidium iodide staining and FACS (data not shown).
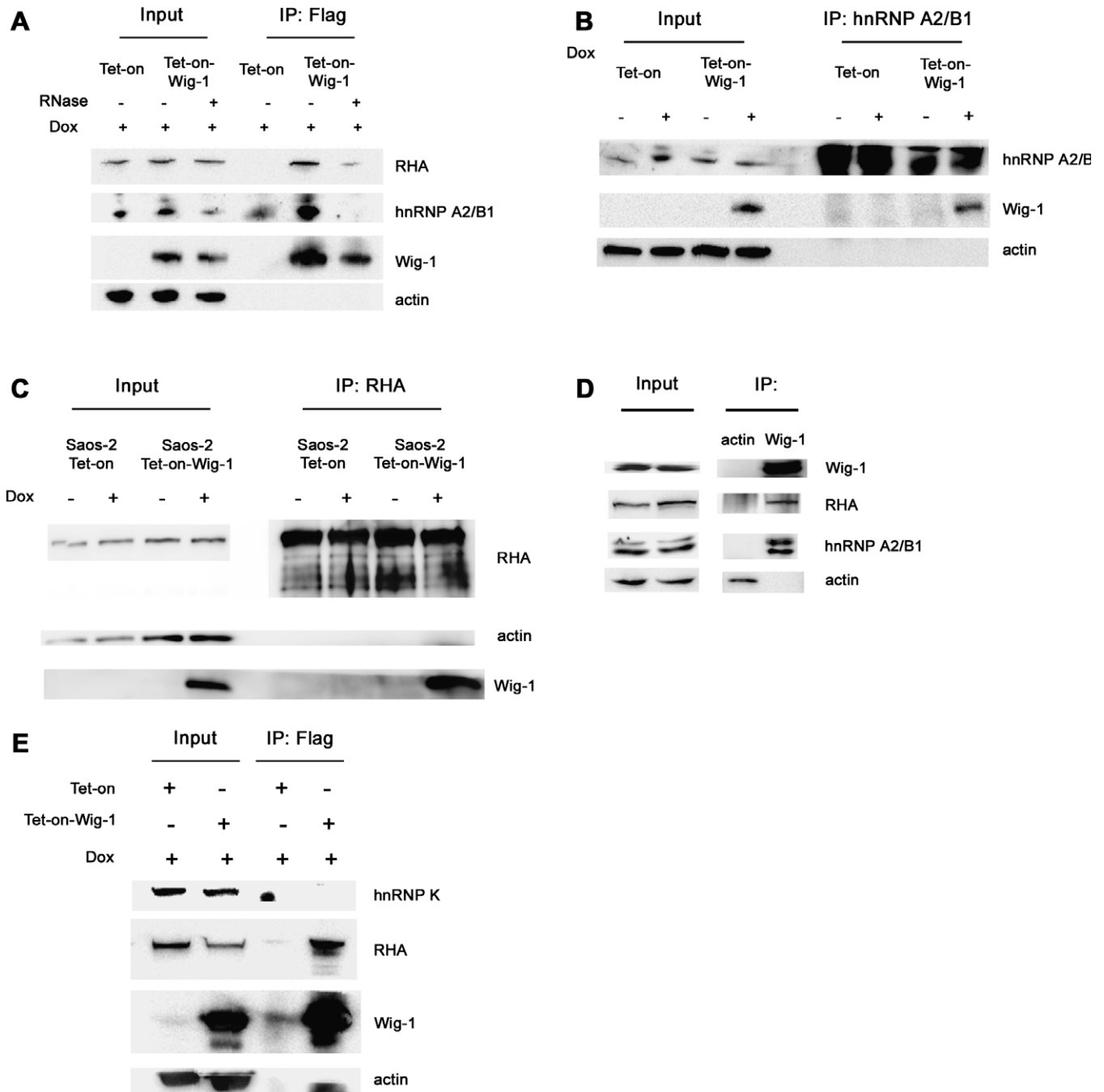

Fig. 2. Wig-1 binds hnRNP A2/B1 and RHA, but not hnRNPK, via RNA. (A) Protein lysates from Saos-2 Tet-on and Saos-2 Tet-on-Wig-1 cells cultured in the presence or absence of doxycycline were treated with RNase followed by immunoprecipitation with Flag antibody and Western blotting using indicated antibodies. (B, C) Protein lysates from Saos-2 Tet-on and Saos-2 Tet-on-Wig-1 cells cultured in the presence or absence of doxycycline were immunoprecipitated with an hnRNP A2/B1 antibody (B) or an RHA antibody (C) followed by Western blotting using indicated antibodies. (D) Protein lysates from MCF7 cells were immunoprecipitated with β-actin or RHA antibodies followed by Western blotting with indicated antibodies. (E) Protein lysates from Saos-2 Tet-on and Saos-2 Tet-on-Wig-1 cells were immunoprecipitated with a Flag antibody followed by Western blotting with indicated antibodies.

3.3. Identification of Wig-1-interacting proteins

To identify Wig-1-binding proteins, we immunoprecipitated Wig-1 using Flag antibody, separated proteins on an SDS polyacrylamide gel, and visualized protein bands by silver staining. Several proteins were co-immunoprecipitated with Wig-1. The two most prominent proteins with apparent molecular weights of 38 and 140 kDa were cut out from a Coomassie-stained gel and identified as hnRNP A2/B1 (36/38 kDa) and RNA Helicase A (RHA) (142 kDa) by MALDI mass spectrometry.

3.4. Wig-1 interacts with hnRNPA2/B1 and RHA via RNA

To verify binding, we immunoprecipitated Wig-1, hnRNP A2/B1 or RHA from Saos-2 Tet-on-Wig-1 cells and analysed co-immunoprecipitating proteins by Western blotting (Fig. 2A–C). We also found that the endogenous Wig-1, hnRNP A2/B1 and RHA proteins form complexes in MCF7 cells (Fig. 2D) and HEK293 cells (data not shown). The fact that all three proteins bind dsRNA in living cells suggested that they might interact through RNA. To investigate this, we treated lysates with RNase A prior to immunoprecipitation. This treatment almost completely abolished the binding (Fig. 2A). We conclude that the interaction of Flag-Wig-1 with hnRNP A2/B1 and RHA is dependent on RNA. As a control for

unspecific RNA-mediated interactions, we assessed Wig-1 binding to the abundant RNA-binding protein hnRNP K. Wig-1 did not bind hnRNP K (Fig. 2E).

3.5. The ZF1 and ZF2 point mutants do not bind RHA or hnRNP A2/B1

The first and the second zinc finger domains of Wig-1 are necessary for RNA binding in living cells [4]. To test if the ZF1 and ZF2 mutants can bind hnRNP A2/B1 and/or RHA, we performed immunoprecipitation experiments in the Saos-2 Tet-on-ZF1pm and Saos-2 Tet-on-ZF2pm cells. Neither mutant co-immunoprecipitated with RHA and hnRNP A2/B1 (Fig. 3A), confirming that the interaction between Wig-1 and hnRNP A2/B1 and RHA is dependent on RNA.

3.6. RHA and hnRNP A2/B1 interact via RNA

Finally, we tested if hnRNP A2/B1 and RHA were able to interact with each other via RNA. As shown in Fig. 3B, hnRNP A2/B1 co-immunoprecipitated with the RHA antibody in HEK293 cells. The binding was abolished by RNase A. Thus, hnRNP A2/B1 and RHA form a complex in HEK293 cells which is dependent on RNA.
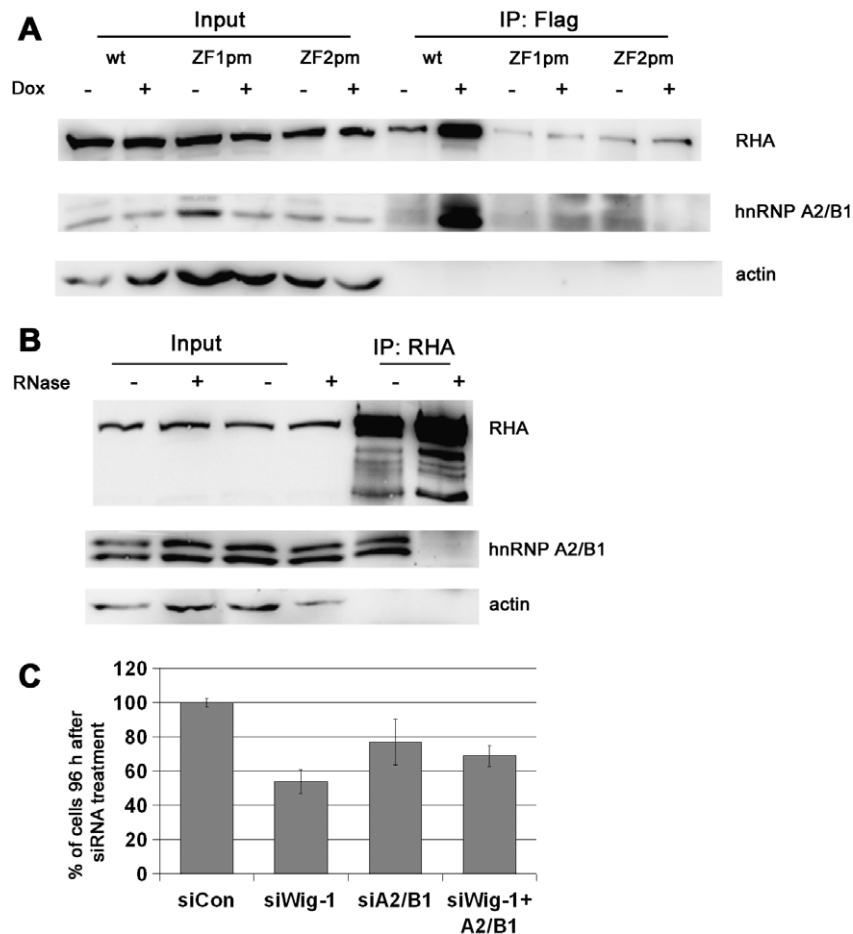

Fig. 3. ZF1 and ZF2 point mutant forms of Wig-1 do not bind hnRNP A2/B1 nor RHA. (A) Protein lysates from Saos-2 Tet-on-Wig-1, Saos-2 Tet-on-ZF1pm and Saos-2 Tet-on ZF2pm cells cultured in the presence or absence of doxycycline were immunoprecipitated with Flag antibody followed by Western blotting using indicated antibodies. (B) hnRNP A2/B1 and RHA interact with each other. A protein lysate from HEK293 cells was treated with RNase followed by immunoprecipitation with RHA and Western blotting with indicated antibodies. (C) HCT116 cells were plated and transfected with the indicated siRNA in 96-well plates in triplicates and analysed after 96 h for growth suppression using the WST-1 cell proliferation reagent. The graph shows percentage of cells compared to siCon.

3.7. Knockdown of Wig-1 and hnRNP A2/B1 inhibits cell growth

To examine the effect of Wig-1 knock-down, we treated HCT116 colon carcinoma cells carrying wild-type p53 with siRNA against Wig-1. Wig-1 knock-down caused a marked growth inhibition as judged by the WST-1 proliferation assay (Fig. 3C). Knockdown of hnRNP A2/B1 shows a similar, albeit slightly less pronounced effect, as previously reported [9]. Interestingly, the simultaneous knockdown of both proteins had a comparable effect as knockdown of either of the two, suggesting that they act in the same pathway (Fig. 3C). We obtained similar data in Saos-2 cells using FACS-PI to assess the sub-G1 (apoptotic) cell fraction. After 72 h of siRNA treatment, a significant difference was observed between siControl and siWig-1, sihnRNP A2/B1, or siWig-1+ sihnRNP A2/B1, whereas no significant differences were observed between the latter three in terms of fraction of sub-G1 cells after treatment (data not shown).

4. Discussion

The fact that Wig-1 is a p53 target gene [1,4,5,14] suggests that it is involved in the p53 response to cellular stress. We have previously demonstrated that Wig-1 inhibits cell growth in a colony formation assay [1,4]. In order to further analyse the effects of Wig-1 expression in the absence of p53, we generated Saos-2 (p53 null) cells carrying tetracycline-regulated human Wig-1. Our WST-1 assay demonstrated that Wig-1 has a modest effect on cell growth. Surprisingly, we did not detect any effect of Wig-1 on cell cycle distribution or any increased fraction of cells with sub-G1 DNA content. This shows that Wig-1 does not inhibit cell growth by blocking cell cycle progression or by triggering a major apoptotic response.

Our co-immunoprecipitation experiments identified hnRNP A2/B1 and RHA as protein partners of Wig-1. This binding was confirmed in HEK293 and MCF7 cells expressing endogenous Wig-1. It requires RNA, since RNAse treatment abolishes binding. Furthermore, the ZF1 and ZF2 point mutants, which are deficient for RNA binding in living cells, do not interact with hnRNP A2/B1 and RHA, and are also unable to inhibit cell growth. These findings suggest that Wig-1-mediated growth inhibition is somehow dependent on the binding to hnRNP A2/B1 and RHA.

It remains to be elucidated which (groups of) dsRNA Wig-1 binds to in living cells. Therefore we can only speculate on the possible link between the Wig-1 dsRNA binding and cell growth and/or survival, and the roles of hnRNP A2/B1 and RHA in these processes. We have shown here that both overexpression and silencing of Wig-1 can inhibit cell growth. Interestingly, downregulation of hnRNP A2/B1 also inhibits cell growth without causing apoptosis [9]. Furthermore, we show that knockdown of both Wig-1 and hnRNP A2/B1 simultaneously results in the same growth inhibitory effect as silencing either one alone, indicating that these two proteins function in the same pathway. We speculate that p53 might block hnRNP A2/B1 through Wig-1, thus inhibiting cell proliferation by a previously unknown mechanism.

Our finding that Wig-1 interacts with RHA via RNA raises the possibility that Wig-1 exerts its growth inhibitory effects by interfering with transcription, by acting on mRNA processing at some level, or by interfering with the effect of RHA on translation of target mRNAs. Additionally, it is conceivable that Wig-1 and RHA and/or hnRNP A2/B1 are part of protein–RNA complexes that block transport of specific mRNAs across the nuclear membrane, thereby inhibiting their translation into protein.

Our results provide a link between the p53 tumor suppressor pathway and RNA processing via hnRNP A2/B1 and RHA. p53, hnRNP A2/B1 and RHA are all multifunctional proteins that are important for the survival of the cell. Therefore it is plausible that they are functionally connected. Further studies of the Wig-1–hnRNP A2/B1–RHA interactions will hopefully provide a better understanding of the function of Wig-1 in the p53 tumor suppressor pathway and the possible role of hnRNP A2/B1 and RHA in regulation of cell growth and survival.

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