# Importin 7 and Exportin 1 Link c-Myc and p53 to Regulation of Ribosomal Biogenesis

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# Importin 7 and exportin 1 link c-Myc and p53 to regulation of ribosomal biogenesis

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## Summary

Members of the β-karyopherin family mediate nuclear import of ribosomal proteins and export of ribosomal subunits, required for ribosome biogenesis. We report that transcription of the β-karyopherin genes *importin 7* (*IPO7*) and *exportin 1* (*XPO1*), and several additional nuclear import receptors, is regulated positively by c-Myc and negatively by p53. Partial IPO7 depletion triggers p53 activation and p53-dependent growth arrest. Activation of p53 by *IPO7* knockdown has distinct features of ribosomal biogenesis stress, with increased binding of Mdm2 to ribosomal proteins L5 and L11 (RPL5 and RPL11). Furthermore, p53 activation is dependent on RPL5 and RPL11. Of note, *IPO7* and *XPO1* are frequently overexpressed in cancer. Altogether, we propose that c-Myc and p53 counter each other in the regulation of elements within the nuclear transport machinery, thereby exerting opposing effects on the rate of ribosome biogenesis. Perturbation of this balance may play a significant role in promoting cancer.

## Introduction

The link between ribosome biogenesis and cancer has been revealed more than a century ago, when the cellular site of ribosome biogenesis, the nucleolus, was found to be morphologically distinct between transformed and normal cells (Montanaro et al., 2008; Pianese, 1896). For many years it was perceived that the excessive protein synthesis and the alterations in the protein synthesis machinery are merely reflections of the uncontrolled proliferation of malignant cells. However, more recent evidence clearly established that ribosome biogenesis is highly regulated. Many components of the protein synthesis machinery are either deregulated or mutated in multiple forms of cancer (Ruggero and Pandolfi, 2003). Thus, deregulated expression of ribosomal proteins (RP) is observed in tumors and in cancer-derived cell lines (Ferrari et al., 1990; Kondoh et al., 2001; Pogue-Geile et al., 1991), and forced overexpression of RPS3a was sufficient to transform NIH3T3 mouse fibroblasts and induce the formation of tumors in nude mice (Naora et al., 1998). Not only RP, but also other factors in the ribosome biogenesis pathway, such as NPM/B23 (Lim and Wang, 2006) and DKC1 (Ruggero et al., 2003), are often deregulated in cancer.

The relationship between cancer and ribosome biogenesis is also well reflected by the activity of the proto-oncogene c-Myc. One of the best studied cancer-promoting proteins, c-Myc is a key player in multiple types of cancer (Albihn et al., 2010; Gustafson and Weiss,

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2010). A master regulator of cell proliferation, growth, metabolism and differentiation (Eilers and Eisenman, 2008; Soucek and Evan, 2010), c-Myc is also deeply involved in ribosome biogenesis. c-Myc is a co-activator of RNA polymerase (RNA Pol) I and III in the transcription of rRNA (Gomez-Roman et al., 2003; Grandori et al., 2005). Its role in regulating the transcription of genes encoding ribosomal proteins and other accessory factors of the ribosome biogenesis machinery is also well established (Ruggero, 2009; van Riggelen et al., 2010).

Probably due to the central role of ribosome biogenesis in cell growth and proliferation, several tumor suppressors such as pRB, p53 and PTEN closely inspect the fidelity of this process. Low concentrations of actinomycin D that inhibit selectively the synthesis of ribosomal RNA (rRNA) by Pol I, effectively activate p53 (Choong et al., 2009). Similar effects were observed upon depletion of TIF-IA, a positive regulator of rRNA synthesis by Pol I (Yuan et al., 2005). Partial depletion of several ribosomal proteins including RPS6, RPS9, RPL23, RPL24, RPL29 and RPL30, also induces a p53-mediated stress signal (Barkic et al., 2009; Lindstrom and Nister, 2010; Panic et al., 2006; Sun et al., 2010). Notably, upon ribosomal biogenesis stress several ribosomal proteins associate with Mdm2, a key negative regulator of p53: this leads to Mdm2 inactivation and consequently p53 activation (Bhat et al., 2004; Dai and Lu, 2004; Jin et al., 2004; Lohrum et al., 2003; Marechal et al., 1994; Yadavilli et al., 2009; Zhang et al., 2010; Zhu et al., 2009). It is generally believed that when ribosome biogenesis is disrupted, unassembled RP are released from the nucleolar ribosome assembly “factories”, thus becoming free to bind Mdm2 and activate p53 (Zhang and Lu, 2009). Alternatively, ribosomal biogenesis stress can increase total RPL11 levels by augmenting the translation of *RPL11* mRNA, thereby also leading to Mdm2 inactivation and p53 stabilization (Fumagalli et al., 2009). In line with its role in regulating ribosome biogenesis, p53 actively represses the transcription of rRNA through direct binding to the Pol I complex (Budde and Grummt, 1999; Zhai and Comai, 2000).

Before being assembled into ribosomal subunits within the nucleolus, freshly translated RP need to translocate from the cytoplasm, their site of production, into the nucleus. In fact, RP possess a very short half life (2–3 minutes) in the cytoplasm, and accumulate in the nucleolus almost immediately after their synthesis (Warner et al., 1985). Although, in theory, the average RP size is well below the diffusion limit through the nuclear pore, in reality RP import is energy dependent and requires the assistance of nuclear import factors. Several nuclear receptors (importins), members of the β-Karyopherin family, participate in this active nuclear import process (Jakel and Gorlich, 1998; Jakel et al., 2002; Rout et al., 1997). Additionally, β-Karyopherins prevent the aggregation of ribosomal proteins with other polyanionic molecules (Jakel et al., 2002). To date, several importins were reported to participate in the nuclear import of RP (Jakel and Gorlich, 1998; Plafker and Macara, 2002; Rout et al., 1997); one of those is importin 7 (*IPO7*), whose cargo comprises RPL5, RPL4, RPL6 and RPL23A, and presumably additional RP. IPO7 can also transport non-RP cargoes, including histone H1 (Jakel et al., 1999), EZI (Saijou et al., 2007), the HIV RTC complex (Fassati et al., 2003) and others.

LIttle is known about how the levels of *IPO7* and other import factors are regulated. Notably, *IPO7* is overexpressed in colorectal cancer (Li et al., 2000) and elevated *IPO7* was also found in transformed mammary and ovarian cells (Smith et al., 2010). In view of the overexpression of *IPO7* in cancer cells, we sought to investigate the molecular mechanisms governing its expression as well as to elucidate the possible contribution of *IPO7* overexpression to tumor cell properties. We now report that the *IPO7* gene is a positive transcriptional target of c-Myc, presumably accounting for its upregulation in colorectal cancer. Conversely, *IPO7* transcription is repressed by p53. A very similar mode of regulation applies to exportin 1 (*XPO1/CRM1*), required for the export of assembled

ribosomal subunits from the nucleolus back into the cytoplasm. Thus, c-Myc governs not only the synthesis of the various ribosomal components but also the machinery responsible for their proper intracellular translocation at different stages of ribosomal biogenesis. Conversely, p53 inhibits ribosomal biogenesis by downregulating critical components of the transport machinery. Notably, IPO7 depletion elicits ribosomal biogenesis stress, leading to p53 activation via association of Mdm2 with RPL5 and RPL11, with subsequent growth arrest. Loosening the tight regulation of this process may promote cancer.

## Results

### The *IPO7* gene is a c-Myc target

c-Myc overexpression is very common in colorectal cancer (CRC), due to *APC* mutations and excessive Wnt/β-catenin signaling (Fearon and Dang, 1999; He et al., 1998). Because *IPO7* is overexpressed in CRC and its levels correlate with those of c-Myc (Li et al., 2000), we asked whether the *IPO7* gene is transcriptionally regulated by c-Myc. To that end we transiently knocked down c-Myc in two CRC cell lines, HCT116 and HT-29, using specific siRNA, and assessed *IPO7* expression. Indeed, partial c-Myc depletion moderately downregulated *IPO7* mRNA (Fig 1A, upper panel) and protein (Fig. 1B). The decrease in IPO7 protein was validated with two different c-Myc siRNA oligonucleotide combinations (Fig S1D). Nuclear export of the 40S and 60S ribosomal subunits is dependent on XPO1/CRM1 (Thomas and Kutay, 2003). *XPO1*, too, is overexpressed in multiple types of cancer (Huang et al., 2009; Shen et al., 2009; van der Watt et al., 2009). Remarkably, *XPO1* mRNA decreased significantly following c-Myc knockdown (Fig 1A, lower panel). To determine whether c-Myc can upregulate *IPO7* and *XPO1* also in normal cells, we analyzed mouse embryonic fibroblasts (MEF) 72 hours after infection with a recombinant retrovirus encoding human c-Myc. Indeed, c-Myc overexpression upregulated both *IPO7* and *XPO1* mRNA (Fig. 1C) and IPO7 protein (Fig 1D). Analysis of the human *IPO7* and *XPO1* genes revealed putative c-Myc binding sites (E-box) in the core promoter of *IPO7* and the first intron of *XPO1* (Fig S1A). Chromatin immunoprecipitation (ChIP) with specific c-Myc antibodies confirmed c-Myc association with the corresponding genomic regions (Fig. 1E,F4) in both HCT116 and HT-29 cells. *In silico* analysis identified putative E-Box consensus sites in several additional genes encoding beta-karyopherin family members (Fig. S1A). Furthermore, partial c-Myc depletion reduced *IPO5* mRNA levels in HCT116 cells (Fig. S1B), whereas c-Myc overexpression in MEF increased the transcript levels of *IPO4*, *IPO5*, *IPO11* and transportin 1 (*TNPO1*) (Fig. S1C). Additionally, c-Myc overexpression upregulated practically all RP mRNAs (Table S1); the upregulation was mostly moderate, while L7, L38, S4, S9, S24, L8 and L37A were more strongly induced. Conceivably, in order to deal effectively with the increase in ribosomal biogenesis, c-Myc overexpressing cells upregulate also components of the machinery responsible for the nuclear import of ribosomal proteins and the export of assembled ribosomal subunits.

### IPO7 depletion reduces the nuclear import of RPL4

Several beta-karyopherins have been implicated in the nuclear import of ribosomal proteins (Jakel and Gorlich, 1998; Jakel et al., 2002; Plafker and Macara, 2002). *In vitro* experiments suggested that multiple import proteins redundantly mediate the import of the same RP, raising the question whether depletion of IPO7 alone is sufficient to attenuate RP import. Along with importin β1 (KPNB1), IPO7 was found to be the most efficient carrier for the very basic RPL4 and RPL6 (Jakel et al., 2002); the IPO7/KPNB1 heterodimer is probably the physiological receptor for nuclear import of RPL4 (Jakel et al., 2002). To assess the impact of IPO7 depletion on RP import in live cells, we therefore employed FRAP (Fluorescence Recovery After Photobleaching) to measure the dynamics of RPL4 nuclear import, as described before (Lam et al., 2007). We took advantage of a clone of H1299 cells

endogenously expressing YFP-tagged RPL4 (Cohen et al., 2008; Frenkel-Morgenstern et al., 2009). As seen in Fig. 2A,B, IPO7 depletion significantly reduced the accumulated levels of fluorescent RPL4-YFP in the photobleached nucleoli, even though the total amount of RPL4 was unaffected (Fig 2C). This implies that IPO7 is imperative for the nuclear accumulation of RPL4, and perhaps of additional client RPs.

### IPO7 depletion leads to changes in nucleolar morphology

A variety of cellular insults affecting ribosomal biogenesis lead to altered nucleolar structure (Boulon et al., 2010). Using antibodies specific for the nucleolar proteins fibrillarin and UBF, we noted subtle changes in the morphology of the nucleoli in HCT116 cells depleted of IPO7, resulting in a more condensed appearance (Fig 3). Similar changes were observed upon inhibition of rRNA transcription or early rRNA processing (Burger et al., 2010). Altered nucleolar morphology upon IPO7 knockdown was also observed by staining for another nucleolar protein, B23 (Fig S2A), as well as after XPO1 knockdown (Fig. S2A). Furthermore, in non-transformed MRC5 fibroblasts IPO7 depletion gave rise to a more fragmented B23 staining pattern, with an overall decrease in B23 staining intensity (Fig. S2B). These observations are consistent with the notion that inhibition of ribosomal protein import and export elicits nucleolar alterations and presumably nucleolar stress.

### IPO7 depletion triggers p53 activation

Ribosome biogenesis is an essential element of cell growth and is tightly coupled with cell proliferation (Ruggero and Pandolfi, 2003). To assess the effect of IPO7 depletion on cell proliferation, cultures were subjected to analysis of BrdU incorporation. IPO7 knockdown decreased the percentage of BrdU-positive cells in both HCT116 and MRC5 cells (Fig S3A). Notably, this was not observed in p53-deficient HCT116 cells, nor in p53-depleted MRC5 cells (Fig S3A).

The inhibitory effect of IPO7 depletion was further validated in a colony formation assay. As seen in Fig 4A, depletion of IPO7 in WT HCT116 cells (p53+/+) led to a strong reduction in colony numbers. In contrast, IPO7 knockdown had only a very mild effect in HCT116 cells with a somatic p53 knockout (p53−/−). Furthermore, the reduction in colony number in WT HCT116 cells was practically abrogated by concomitant transient knockdown of p53 (lower left panel). Hence, the antiproliferative effect of IPO7 depletion requires functional p53.

Remarkably, IPO7 depletion in HCT116 and MRC5 cells led to p53 elevation (Fig 4B). Accordingly the CDK inhibitor p21, a p53 transcriptional target, was induced at both protein and mRNA levels (Fig 4B–D) in a p53-dependent manner (Fig. 4C,D). Thus, IPO7 depletion triggers p53 activation.

Likewise, depletion of importin β1(KPNB1), but not IPO13, also resulted in p53 accumulation and p21 induction, albeit to a lesser extent than IPO7 (Fig S3B,C). The most robust p53 response was observed upon XPO1 knockdown; this is not surprising, since the XPO1 inhibitor Leptomycin B also triggers extensive p53 induction (Freedman and Levine, 1998).

### IPO7 depletion activates p53 through ribosomal biogenesis stress

The p53/Mdm2 axis is highly responsive to ribosomal biogenesis stress (Lu 2009). Accumulation of excessive unassembled ribosomal proteins RPL11 and RPL5 plays a critical role. Notably, induction of p53 upon IPO7 knockdown (Fig 5A, lanes 1,2) was completely blunted by concomitant depletion of RPL11 (lane 4) or RPL5 (Fig 5B). Activation of p53 occurs through interaction of RPL11 with Mdm2: under conditions of

ribosomal biogenesis stress unassembled RPL11 binds Mdm2 and prevents p53 degradation (Lohrum et al., 2003; Zhang and Lu, 2009). Indeed, a robust interaction between Mdm2 and RPL11 was seen in HCT116 cells depleted of IPO7 (Fig. 5C, lane 8). To confirm this was not merely due to induction of the endogenous Mdm2 upon p53 activation, the analysis was repeated with exogenously expressed Mdm2. Again, more RPL11 coprecipitated with Mdm2 following IPO7 depletion (Fig. S4, compare lanes 5 and 6). Similar results were obtained with RPL5 (Fig. S4). Together, these observations argue strongly that IPO7 depletion elicits ribosomal biogenesis stress, triggering p53 activation.

## p53 represses *IPO7* and *XPO1* gene expression

Interestingly, transient knockdown of p53 in MRC5 cells caused an increase in *IPO7* (Fig 6A) and *XPO1* (Fig 6B) mRNA, suggesting negative regulation of both genes by p53. Further support for this conjecture was obtained with MEF. As expected, c-Myc overexpression in wild type (wt) MEF caused upregulation of *IPO7* (Fig. 6C). Yet, both basal and c-Myc-induced *IPO7* mRNA (Fig. 6C) and protein (Fig. 6D) were higher in MEF derived from p53-null mice when compared to wt MEF, Essentially similar results were obtained with *XPO1* mRNA (Fig 6E). Hence, p53 represses basal *IPO7* and *XPO1* expression and attenuates their induction by c-Myc.

These observations imply that basal p53 activity is sufficient to repress *IPO7* and *XPO1* in non-stressed cells. To determine whether p53 activation under conditions of ribosomal biogenesis stress further attenuates the expression of both genes, MRC5 cells were exposed to a low concentration of actinomycin D (ActD), with or without p53 knockdown. As expected, both *IPO7* and *XPO1* mRNA were upregulated following p53 knockdown (Fig. 6F). Notably, ActD elicited a moderate decrease of both transcripts, which was at least partially reversed by p53 knockdown. Importantly, analysis of heterogeneous nuclear RNA (hnRNA) with intronic PCR primers (Fig. 6F, Intronic), which provides an approximation of transcription rates (Kuroda et al., 2005; Phelps et al., 2006), confirmed that the repression of *IPO7* and *XPO1* by both basal and ActD-activated p53 occurred at the transcriptional level.

The *IPO7* and *XPO1* genes (Fig S1A) harbor sequences matching closely the proposed consensus for p53-mediated transcriptional repression (Wang et al, 2009). *IPO7* harbors two such sites, one in the proximal promoter and one within the first intron, approximately 4Kb downstream to the transcription start site (TSS). In *XPO1*, a single putative site resides about 600 nucleotides upstream to the TSS. ChIP analysis confirmed p53 binding to the corresponding regions, further augmented by ActD treatment (Fig 7A and 7B).

To further explore the regulation of *IPO7* by c-Myc and p53, the *IPO7* promoter was cloned into a luciferase reporter (Fig S5A). As expected, transfected p53 repressed *IPO7* promoter activity in p53-null H1299 cells (Fig S5B). Moreover, promoter activity was higher in p53-deficient HCT116 cells than in cells harboring wt p53 (Fig S5D). However, deletion of the putative p53 response element (p53-RE) failed to abolish p53-mediated repression (Fig S5B); hence, at least in the context of non-chromosomal plasmid DNA, this element is not required for repression.

p53 represses transcription through a variety of mechanisms (Menendez et al., 2009; Wang et al., 2010). One indirect mechanism operates through p21 (Gottifredi et al., 2001; Shats et al., 2004). Indeed, ActD was unable to downregulate IPO7 mRNA (Fig S5C upper panel) or protein (Fig S5C lower panel) in p21-deficient HCT116 cells. Thus, despite the direct binding of p53 to the IPO7 gene, much of the impact of p53 on *IPO7* expression may be exerted via p21.

A similar approach was taken to address the positive regulation of *IPO7* by c-Myc. Indeed, transfected c-Myc activated the *IPO7* promoter, particularly in p53-deficient cells (Fig. S5D). Unexpectedly, deletion of the E-Box did not abrogate transactivation by c-Myc. It remains possible that this element is operative only when the *IPO7* promoter resides within chromatin.

In conclusion, p53 represses genes encoding proteins involved in both nuclear import of ribosomal proteins and the of assembled ribosomal subunits back into the cytoplasm. Moreover, p53 restricts the positive effects of c-Myc on those genes.

## Discussion

### Regulation of importin 7 and exportin 1 by c-Myc and p53

We report that importin 7 (IPO7) and exportin 1 (XPO1/CRM1), as well as several additional genes involved in nuclear import and export, are regulated positively by c-Myc and negatively by p53. The effects are mostly modest; however, given that IPO7 and XPO1 are abundant proteins whose levels are tightly controlled, even modest changes may have substantial impact on the efficiency of nuclear import and export and thereby on cellular phenotype.

The exact mechanisms whereby p53 and c-Myc regulate these genes remains unclear. Both *IPO7* and *XPO1* carry putative p53 and c-Myc binding sites, and both transcription factors bind the corresponding regions within chromatin. Moreover, the *IPO7* and *XPO1* promoters are regulated positively by c-Myc and negatively by p53. Yet, contrary to expectation, deletion of the putative binding sites does not seem to affect transcriptional regulation. It remains possible that the putative sites are indeed functional, but only within the context of chromatin. Alternatively, and not mutually exclusive, indirect mechanisms may be in operation. In the case of *IPO7*, our data suggest a role for p21 in such indirect effect; this may involve a putative E2F site, located at positions 14–22 relative to the TSS (not shown). As for *XPO1*, a recent study showed that p53 represses its expression through binding to NF-Y (van der Watt and Leaner, 2011). As the NF-Y sites resides in close proximity to the putative p53-RE, it is plausible that the observed binding of p53 is mediated through NF-Y rather than through direct binding to the predicted p53-RE. Yet, regardless of mechanism, our findings argue that the opposing effects of p53 and c-Myc on components of the nuclear import/export machinery have notable biological consequences.

### c-Myc as a regulator of ribosome import/export

As most of the ribosome assembly process takes place in the nucleolus, the import of ribosomal proteins (RP) into the nucleus as well as export of assembled ribosomal subunits back into the cytoplasm are prerequisites for successful execution of this process. We find that c-Myc, a master regulator of ribosome biogenesis (van Riggelen et al.), positively regulates also the expression of β-karyopherin involved in the import/export machinery. Specifically, c-Myc transactivates the genes encoding IPO7 and XPO1/CRM1, which participate in the import and export, respectively, of RP and ribosomal subunits (Jakel and Gorlich, 1998; Johnson et al., 2002). Excessive c-Myc activity is a common oncogenic driver in many types of cancer. Not surprisingly, both *IPO7* and *XPO1* are overexpressed in various human tumors (Huang et al., 2009; Li et al., 2000; van der Watt et al., 2009). While the role of *XPO1* in cancer has been thoroughly explored (Huang et al., 2009; Shen et al., 2009; Turner and Sullivan, 2008), the role of *IPO7* remains largely unknown.

The *IPO7* and *XPO1* genes appear to be upregulated by c-Myc as part of a coordinated transcriptional program that also includes practically all RP genes (Wu et al., 2008)(see also Supplemental Table S1). The c-Myc-induced increase in RP levels is expected to impose an

increased burden on the nuclear transport machinery; the simultaneous increase in IPO7 and XPO1 presumably aims to cope with this burden and maintain cellular homeostasis. Another relevant c-Myc target is nucleophosmin (NPM1/B23) (Zeller et al., 2001), which regulates the nuclear export of RP and ribosomal subunits (Maggi et al., 2008; Yu et al., 2006), presumably through interaction with XPO1/CRM1. Interestingly, excessive c-Myc activity can also induce the tumor suppressor ARF (Zindy et al., 1998), which, in turn, inhibits c-Myc activity independently of its p53-activating role (Qi et al., 2004). Of note, ARF sequesters B23 in the nucleolus, thereby impeding the shuttling of B23 and the RPL5-5S complex to the cytoplasm (Brady et al., 2004; Yu et al., 2006). Moreover, ARF also regulates Mdm2 export (Tao and Levine, 1999). Thus, ARF may protect normal cells against illegitimate c-Myc hyperactivation by restricting the shuttling of ribosomal components.

### Depletion of IPO7 leads to p53 activation

As shown here, depletion of *IPO7* results in p53 activation and subsequent growth inhibition. Of note, one of the reported cargoes of IPO7, RPL5, participates in the p53 response to ribosomal stress (Dai and Lu, 2004; Zhu et al., 2009). It is possible that IPO7 depletion causes imbalance in the composition of RP within the nucleolus, causing those RP that are not IPO7-dependent to accumulate in unassembled excess. These may include RPL11, which then would bind Mdm2 and induce p53 activation. Alternatively, since binding of RPL5 and RPL23 to Mdm2 occurs in both the nucleus and the cytoplasm (Dai and Lu, 2004; Dai et al., 2004), IPO7 depletion may cause complete or partial retention of unassembled RPL5 in the cytoplasm, where it is free to inhibit cytoplasmic Mdm2. Either way, the p53 connection may underlie the successful generation of clones harboring stable IPO7 knockdown (Zaitseva et al., 2009), as both cell lines employed in that study, HeLa and Jurkat, lack functional p53.

### p53-mediated repression of the import/export machinery

Our findings imply that p53 represses the import/export machinery as part of programmed inhibition of ribosome biogenesis. Indeed, exposure of HCT116 cells to genotoxic stress alters the nuclear/cytoplasmic distribution of RP in a p53-dependent manner (Boisvert and Lamond, 2010).

It has been suggested that p53 is activated only upon nucleolar damage (Rubbi and Milner, 2003), and that inhibition of nucleolus-dependent p53 export is a major activating mechanism. Of note, p53 and Mdm2 are found associated with RPL5 and 5S rRNA (Marechal et al., 1994). Moreover, Mdm2 was reported to utilize the same export machinery as do HIV-Rev proteins (Roth et al., 1998), which also relies partly on the export of RPL5 (Schatz et al., 1998). Interestingly, the nuclear import of the HIV reverse transcriptase complex is also mediated by IPO7 (Fassati et al., 2003; Zaitseva et al., 2009). Blocking nuclear export with leptomycin B, an inhibitor of XPO1/CRM1, induces p53 (Freedman and Levine, 1998). Together, this suggests a scenario in which the p53/Mdm2 complex utilizes the nuclear export of RPL5 for its own export, leading to subsequent degradation of p53 (and perhaps Mdm2) in the cytoplasm. Thus, by inhibiting the import/export of RP, p53 may not only inhibit ribosome maturation but also increase its own retention in the nucleus, defining a positive feedback loop.

An interesting implication of our findings is that this new activity of p53 may also play a protective role against viral infection. Many viruses manipulate the nucleolus for their replication and activity (Greco, 2009). By reacting to the nucleolar stress imposed by viral proteins, p53 may inhibit the import and export of viral components, including viral genomes, which rely on the same nuclear import/export machinery that translocates RP

(Fassati et al., 2003; Neville et al., 1997; Zaitseva et al., 2009). This attractive possibility merits further investigation.

In sum, p53 and c-Myc oppose each other in the regulation of yet another aspect of the complex process of ribosome biogenesis (Fig. 7C). The consequences of controlling the "gate" to the nucleus likely extend beyond the biogenesis of ribosomes, as many other cellular processes require rapid transport of proteins from the cytoplasm to the nucleus and vice versa. Indeed, many tumor suppressor proteins and oncoproteins shuttle back and forth between the two compartments, p53 being one. It is conceivable that transition from normal growth control to cancer entails changes in the rates of nuclear import and export of many additional proteins. Hence, the opposing effects of c-Myc and p53 on these processes may underpin, in part, their abilities to act as oncoprotein and tumor suppressor, respectively.

## Materials and methods

### Cell culture and transfections

All human cell lines were maintained at 37°C in a 5% CO2 humidified incubator. Mouse embryonic fibroblasts (MEF) were maintained at 37°C in a 5% CO2 and 3% O2 humidified incubator (Thermo scientific). Wild type HCT116 $p53^{+/+}$, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ cells (generous gift of B. Vogelstein) and HT-29 cells were grown in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HI-FBS, Sigma), 2 mM L-glutamine, and antibiotics. MRC5 cells were grown in Minimal Essential Medium (MEM) supplemented with 10% non-heat-inactivated FBS (Sigma), MEM-Eagle nonessential amino acids, sodium pyruvate, L-glutamine and antibiotics. H1299 expressing RPL4-HFP (generous gift of U.Alon) were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HI-FBS, Sigma) and antibiotics. MEF were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% heat-inactivated FBS (HyClone) and antibiotics. MCF7 cells were maintained in DMEM supplemented with 10% FBS (GIBCO-BRL), 2 mM L-glutamine, and antibiotics.

For details on the H1299 cells endogenously expressing RPL4-YFP see (Frenkel-Morgenstern et al., 2010; Sigal et al., 2007).

For siRNA transfections, Dharmafect 1 reagent (Dharmacon) was used according to the manufacturer's protocol. SiRNA for *IPO7*, *c-Myc*, *p53*, *RPL11*, *RPL5 AND p21* was purchased from Dharmacon as Smartpools.

### Colony formation assay

Following transfection, cells were trypsinized and re-seeded in six-well plates at a density of 5000 cells/well. For crystal violet staining, plates were washed once with PBS and once with cold methanol, and then incubated in crystal violet solution (0.3% in methanol) for 5 min at −20°C. Plates were subsequently washed twice with double distilled water, air-dried, and scanned using a Canon scanner.

### Western blots analysis

Western blot analysis was performed as described (Aylon et al., 2006). Antibodies directed against the following proteins were used: IPO7 (Gorlich et al., 1997), GAPDH (Chemicon), p53 (mixture of DO1 + PAb1801), c-Myc (Cell Signaling), and Mdm2 (mixture of 4B2, 2A9, and 4B11). Gel quantification was performed using ImageJ (NIH).

### Isolation of total RNA and real-time quantitative PCR (qPCR)

Total RNA was isolated with a NucleoSpin RNA II kit (Macherey-Nagel). A 1.5 μg aliquot of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and random hexamer primers (Amersham). Real-time qPCR was performed using SYBR Green Master Mix (Applied Biosystems) in a StepOnePlus instrument (Applied Biosystems). Primer sequences are detailed under Supplemental Material.

### Coimmunoprecipitation analysis

Cell monolayers were gently washed twice with ice-cold PBS and lysed for 30 min on ice with NP-40 lysis buffer (50 mM Tris·HCl at pH 8.0, 150 mM NaCl, 1.0% NP-40) supplemented with protease inhibitor mix (Sigma) and phosphatase inhibitor cocktail I + II (Sigma). Protein A-Sepharose beads (Repligen), pre-blocked with BSA, were incubated with appropriate antibodies. Lysates were incubated with the bound antibodies for 4 h at 4°C, washed with NP-40 lysis buffer, released from beads by boiling, and resolved by SDS-PAGE.

### Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described (Nelson et al., 2006). Precipitated DNA was subjected to qPCR analysis using gene-specific primer pairs. Primer sequences are detailed under Supplemental Material.

### Immunofluorescence staining

Cells were washed twice with PBS and fixed with 3% paraformaldehyde for 10 minutes. Following fixation, cell were permeabilized with 1% Triton X-100 in PBS for 10 minutes, followed by 30 minute incubation with 3% BSA in PBS. Cells were next incubated for 1 hour with rabbit anti Fibrillarin (Abcam #5821) and mouse anti UBF (Santa Cruz #13125). Both antibodies were used at a dilution of 1:200. After 3 washes with PBS, cells were incubated with an appropriate secondary antibody (goat anti rabbit Alexafluor 488 or rabbit anti mouse Alexafluor 564). Nuclei were counterstained with DAPI.

Images were taken with a Zeiss LSM710 confocal microscope, using an X40 immersion oil objective.

### Fluorescence recovery after photobleaching (FRAP)

H1299 cells expressing YFP-tagged endogenous RPL4 were grown in chambered coverglass (Lab-Tek) and maintained during the experiment inside a mounted incubator (PeCon) under 37°C and 5% $CO_2$ conditions. The experiment was done using the IX81-based Olympus Fluoview 1000D confocal microscope equipped with a SIM scanner, using a X40 (1.35 N.A) oil immersed objective. In brief, the entire nuclear area of 4–5 randomly picked cells at each field of view was marked, and the YFP signal was bleached using an 488nm Argon laser at 100% intensity until less than 5% of the original signal was detected. 10 images were collected at time intervals of 5 minutes, and at each time point pictures of 8 focal plains were taken. For FRAP analysis, 4 focal plains that cover approximately the volume of the nuclei were chosen for measurement. The average intensity of the YFP signal in the nucleus of each cell was measured using ImageJ (NIH imaging), and the values were corrected to the average signal of non bleached nuclei.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

Albihn A, Johnsen JI, Henriksson MA. MYC in oncogenesis and as a target for cancer therapies. Adv Cancer Res. 2010; 107:163–224. [PubMed: 20399964]

Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. Genes Dev. 2006; 20:2687–2700. [PubMed: 17015431]

Barkic M, Crnomarkovic S, Grabusic K, Bogetic I, Panic L, Tamarut S, Cokaric M, Jeric I, Vidak S, Volarevic S. The p53 tumor suppressor causes congenital malformations in Rpl24-deficient mice and promotes their survival. Mol Cell Biol. 2009; 29:2489–2504. [PubMed: 19273598]

Bhat KP, Itahana K, Jin A, Zhang Y. Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation. Embo J. 2004; 23:2402–2412. [PubMed: 15152193]

Boisvert FM, Lamond AI. p53-Dependent subcellular proteome localization following DNA damage. Proteomics. 2010; 10:4087–4097. [PubMed: 21080495]

Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI. The nucleolus under stress. Mol Cell. 2010; 40:216–227. [PubMed: 20965417]

Brady SN, Yu Y, Maggi LB Jr, Weber JD. ARF impedes NPM/B23 shuttling in an Mdm2-sensitive tumor suppressor pathway. Mol Cell Biol. 2004; 24:9327–9338. [PubMed: 15485902]

Budde A, Grummt I. p53 represses ribosomal gene transcription. Oncogene. 1999; 18:1119–1124. [PubMed: 10023689]

Burger K, Muhl B, Harasim T, Rohrmoser M, Malamoussi A, Orban M, Kellner M, Gruber-Eber A, Kremmer E, Holzel M, Eick D. Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. J Biol Chem. 2010; 285:12416–12425. [PubMed: 20159984]

Choong ML, Yang H, Lee MA, Lane DP. Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy. Cell Cycle. 2009; 8:2810–2818. [PubMed: 19657224]

Cohen AA, Geva-Zatorsky N, Eden E, Frenkel-Morgenstern M, Issaeva I, Sigal A, Milo R, Cohen-Saidon C, Liron Y, Kam Z, et al. Dynamic proteomics of individual cancer cells in response to a drug. Science. 2008; 322:1511–1516. [PubMed: 19023046]

Dai MS, Lu H. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J Biol Chem. 2004; 279:44475–44482. [PubMed: 15308643]

Dai MS, Zeng SX, Jin Y, Sun XX, David L, Lu H. Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol Cell Biol. 2004; 24:7654–7668. [PubMed: 15314173]

Eilers M, Eisenman RN. Myc's broad reach. Genes Dev. 2008; 22:2755–2766. [PubMed: 18923074]

Fassati A, Gorlich D, Harrison I, Zaytseva L, Mingot JM. Nuclear import of HIV-1 intracellular reverse transcription complexes is mediated by importin 7. Embo J. 2003; 22:3675–3685. [PubMed: 12853482]

Fearon ER, Dang CV. Cancer genetics: tumor suppressor meets oncogene. Curr Biol. 1999; 9:R62–65. [PubMed: 10021355]

Ferrari S, Manfredini R, Tagliafico E, Rossi E, Donelli A, Torelli G, Torelli U. Noncoordinated expression of S6, S11, and S14 ribosomal protein genes in leukemic blast cells. Cancer Res. 1990; 50:5825–5828. [PubMed: 1697501]

Freedman DA, Levine AJ. Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol Cell Biol. 1998; 18:7288–7293. [PubMed: 9819415]

Frenkel-Morgenstern M, Cohen AA, Geva-Zatorsky N, Eden E, Prilusky J, Issaeva I, Sigal A, Cohen-Saidon C, Liron Y, Cohen L, et al. Dynamic Proteomics: a database for dynamics and localizations of endogenous fluorescently-tagged proteins in living human cells. Nucleic Acids Res. 2009; 38:D508–512. [PubMed: 19820112]

Frenkel-Morgenstern M, Cohen AA, Geva-Zatorsky N, Eden E, Prilusky J, Issaeva I, Sigal A, Cohen-Saidon C, Liron Y, Cohen L, et al. Dynamic Proteomics: a database for dynamics and localizations of endogenous fluorescently-tagged proteins in living human cells. Nucleic Acids Res. 2010; 38:D508–512. [PubMed: 19820112]

Fumagalli S, Di Cara A, Neb-Gulati A, Natt F, Schwemberger S, Hall J, Babcock GF, Bernardi R, Pandolfi PP, Thomas G. Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction. Nat Cell Biol. 2009; 11:501–508. [PubMed: 19287375]

Gomez-Roman N, Grandori C, Eisenman RN, White RJ. Direct activation of RNA polymerase III transcription by c-Myc. Nature. 2003; 421:290–294. [PubMed: 12529648]

Gorlich D, Dabrowski M, Bischoff FR, Kutay U, Bork P, Hartmann E, Prehn S, Izaurralde E. A novel class of RanGTP binding proteins. J Cell Biol. 1997; 138:65–80. [PubMed: 9214382]

Gottifredi V, Karni-Schmidt O, Shieh SS, Prives C. p53 down-regulates CHK1 through p21 and the retinoblastoma protein. Mol Cell Biol. 2001; 21:1066–1076. [PubMed: 11158294]

Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, White RJ. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol. 2005; 7:311–318. [PubMed: 15723054]

Greco A. Involvement of the nucleolus in replication of human viruses. Rev Med Virol. 2009; 19:201–214. [PubMed: 19399920]

Gustafson WC, Weiss WA. Myc proteins as therapeutic targets. Oncogene. 2010; 29:1249–1259. [PubMed: 20101214]

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science. 1998; 281:1509–1512. [PubMed: 9727977]

Huang WY, Yue L, Qiu WS, Wang LW, Zhou XH, Sun YJ. Prognostic value of CRM1 in pancreas cancer. Clin Invest Med. 2009; 32:E315. [PubMed: 20003838]

Jakel S, Albig W, Kutay U, Bischoff FR, Schwamborn K, Doenecke D, Gorlich D. The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1. Embo J. 1999; 18:2411–2423. [PubMed: 10228156]

Jakel S, Gorlich D. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. Embo J. 1998; 17:4491–4502. [PubMed: 9687515]

Jakel S, Mingot JM, Schwarzmaier P, Hartmann E, Gorlich D. Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. Embo J. 2002; 21:377–386. [PubMed: 11823430]

Jin A, Itahana K, O'Keefe K, Zhang Y. Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Mol Cell Biol. 2004; 24:7669–7680. [PubMed: 15314174]

Johnson AW, Lund E, Dahlberg J. Nuclear export of ribosomal subunits. Trends Biochem Sci. 2002; 27:580–585. [PubMed: 12417134]

Kondoh N, Shuda M, Tanaka K, Wakatsuki T, Hada A, Yamamoto M. Enhanced expression of S8, L12, L23a, L27 and L30 ribosomal protein mRNAs in human hepatocellular carcinoma. Anticancer Res. 2001; 21:2429–2433. [PubMed: 11724303]

Kuroda M, Oikawa K, Yoshida K, Takeuchi A, Takeuchi M, Usui M, Umezawa A, Mukai K. Effects of 3-methylcholanthrene on the transcriptional activity and mRNA accumulation of the oncogene hWAPL. Cancer Lett. 2005; 221:21–28. [PubMed: 15797623]

Lam YW, Lamond AI, Mann M, Andersen JS. Analysis of nucleolar protein dynamics reveals the nuclear degradation of ribosomal proteins. Curr Biol. 2007; 17:749–760. [PubMed: 17446074]

Li SR, Gyselman VG, Dorudi S, Bustin SA. Elevated levels of RanBP7 mRNA in colorectal carcinoma are associated with increased proliferation and are similar to the transcription pattern of the proto-oncogene c-myc. Biochem Biophys Res Commun. 2000; 271:537–543. [PubMed: 10799331]

Lim MJ, Wang XW. Nucleophosmin and human cancer. Cancer Detect Prev. 2006; 30:481–490. [PubMed: 17113241]

Lindstrom MS, Nister M. Silencing of ribosomal protein S9 elicits a multitude of cellular responses inhibiting the growth of cancer cells subsequent to p53 activation. PLoS One. 2010; 5:e9578. [PubMed: 20221446]

Lohrum MA, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH. Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell. 2003; 3:577–587. [PubMed: 12842086]

Maggi LB Jr, Kuchenruether M, Dadey DY, Schwope RM, Grisendi S, Townsend RR, Pandolfi PP, Weber JD. Nucleophosmin serves as a rate-limiting nuclear export chaperone for the Mammalian ribosome. Mol Cell Biol. 2008; 28:7050–7065. [PubMed: 18809582]

Marechal V, Elenbaas B, Piette J, Nicolas JC, Levine AJ. The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. Mol Cell Biol. 1994; 14:7414–7420. [PubMed: 7935455]

Menendez D, Inga A, Resnick MA. The expanding universe of p53 targets. Nat Rev Cancer. 2009; 9:724–737. [PubMed: 19776742]

Montanaro L, Trere D, Derenzini M. Nucleolus, ribosomes, and cancer. Am J Pathol. 2008; 173:301–310. [PubMed: 18583314]

Naora H, Takai I, Adachi M, Naora H. Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and suppression of ribosomal protein S3a gene expression induces apoptosis. J Cell Biol. 1998; 141:741–753. [PubMed: 9566973]

Nelson JD, Denisenko O, Bomsztyk K. Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc. 2006; 1:179–185. [PubMed: 17406230]

Neville M, Stutz F, Lee L, Davis LI, Rosbash M. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr Biol. 1997; 7:767–775. [PubMed: 9368759]

Panic L, Tamarut S, Sticker-Jantscheff M, Barkic M, Solter D, Uzelac M, Grabusic K, Volarevic S. Ribosomal protein S6 gene haploinsufficiency is associated with activation of a p53-dependent checkpoint during gastrulation. Mol Cell Biol. 2006; 26:8880–8891. [PubMed: 17000767]

Phelps ED, Updike DL, Bullen EC, Grammas P, Howard EW. Transcriptional and posttranscriptional regulation of angiopoietin-2 expression mediated by IGF and PDGF in vascular smooth muscle cells. Am J Physiol Cell Physiol. 2006; 290:C352–361. [PubMed: 16176970]

Pianese G. Beitrag zur Histologie und Aetiologie der Carcinoma. Histologische und experimentelle. Untersuchungen Beitr Pathol Anat Allgem Pathol. 1896; 142:1–193.

Plafker SM, Macara IG. Ribosomal protein L12 uses a distinct nuclear import pathway mediated by importin 11. Mol Cell Biol. 2002; 22:1266–1275. [PubMed: 11809816]

Pogue-Geile K, Geiser JR, Shu M, Miller C, Wool IG, Meisler AI, Pipas JM. Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. Mol Cell Biol. 1991; 11:3842–3849. [PubMed: 1712897]

Qi Y, Gregory MA, Li Z, Brousal JP, West K, Hann SR. p19ARF directly and differentially controls the functions of c-Myc independently of p53. Nature. 2004; 431:712–717. [PubMed: 15361884]

Roth J, Dobbelstein M, Freedman DA, Shenk T, Levine AJ. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. Embo J. 1998; 17:554–564. [PubMed: 9430646]

Rout MP, Blobel G, Aitchison JD. A distinct nuclear import pathway used by ribosomal proteins. Cell. 1997; 89:715–725. [PubMed: 9182759]

Rubbi CP, Milner J. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. Embo J. 2003; 22:6068–6077. [PubMed: 14609953]

Ruggero D. The role of Myc-induced protein synthesis in cancer. Cancer Res. 2009; 69:8839–8843. [PubMed: 19934336]

Ruggero D, Grisendi S, Piazza F, Rego E, Mari F, Rao PH, Cordon-Cardo C, Pandolfi PP. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science. 2003; 299:259–262. [PubMed: 12522253]

Ruggero D, Pandolfi PP. Does the ribosome translate cancer? Nat Rev Cancer. 2003; 3:179–192. [PubMed: 12612653]

Saijou E, Itoh T, Kim KW, Iemura S, Natsume T, Miyajima A. Nucleocytoplasmic shuttling of the zinc finger protein EZI Is mediated by importin-7-dependent nuclear import and CRM1-independent export mechanisms. J Biol Chem. 2007; 282:32327–32337. [PubMed: 17848547]

Schatz O, Oft M, Dascher C, Schebesta M, Rosorius O, Jaksche H, Dobrovnik M, Bevec D, Hauber J. Interaction of the HIV-1 rev cofactor eukaryotic initiation factor 5A with ribosomal protein L5. Proc Natl Acad Sci U S A. 1998; 95:1607–1612. [PubMed: 9465063]

Shats I, Milyavsky M, Tang X, Stambolsky P, Erez N, Brosh R, Kogan I, Braunstein I, Tzukerman M, Ginsberg D, Rotter V. p53-dependent down-regulation of telomerase is mediated by p21waf1. J Biol Chem. 2004; 279:50976–50985. [PubMed: 15371422]

Shen A, Wang Y, Zhao Y, Zou L, Sun L, Cheng C. Expression of CRM1 in human gliomas and its significance in p27 expression and clinical prognosis. Neurosurgery. 2009; 65:153–159. discussion 159–160. [PubMed: 19574837]

Sigal A, Danon T, Cohen A, Milo R, Geva-Zatorsky N, Lustig G, Liron Y, Alon U, Perzov N. Generation of a fluorescently labeled endogenous protein library in living human cells. Nat Protoc. 2007; 2:1515–1527. [PubMed: 17571059]

Smith ER, Cai KQ, Smedberg JL, Ribeiro MM, Rula ME, Slater C, Godwin AK, Xu XX. Nuclear entry of activated MAPK is restricted in primary ovarian and mammary epithelial cells. PLoS One. 2010; 5:e9295. [PubMed: 20174585]

Soucek L, Evan GI. The ups and downs of Myc biology. Curr Opin Genet Dev. 2010; 20:91–95. [PubMed: 19962879]

Sun XX, Wang YG, Xirodimas DP, Dai MS. Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. J Biol Chem. 2010; 285:25812–25821. [PubMed: 20554519]

Tao W, Levine AJ. P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A. 1999; 96:6937–6941. [PubMed: 10359817]

Thomas F, Kutay U. Biogenesis and nuclear export of ribosomal subunits in higher eukaryotes depend on the CRM1 export pathway. J Cell Sci. 2003; 116:2409–2419. [PubMed: 12724356]

Turner JG, Sullivan DM. CRM1-mediated nuclear export of proteins and drug resistance in cancer. Curr Med Chem. 2008; 15:2648–2655. [PubMed: 18991627]

van der Watt PJ, Leaner VD. The nuclear exporter, Crm1, is regulated by NFY and Sp1 in cancer cells and repressed by p53 in response to DNA damage. Biochim Biophys Acta. 2011

van der Watt PJ, Maske CP, Hendricks DT, Parker MI, Denny L, Govender D, Birrer MJ, Leaner VD. The Karyopherin proteins, Crm1 and Karyopherin beta1, are overexpressed in cervical cancer and are critical for cancer cell survival and proliferation. Int J Cancer. 2009; 124:1829–1840. [PubMed: 19117056]

van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer. 10:301–309. [PubMed: 20332779]

van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. Nat Rev Cancer. 2010; 10:301–309. [PubMed: 20332779]

Wang B, Xiao Z, Ko HL, Ren EC. The p53 response element and transcriptional repression. Cell Cycle. 2010; 9:870–879. [PubMed: 20160511]

Wang B, Xiao Z, Ren EC. Redefining the p53 response element. Proc Natl Acad Sci U S A. 2009; 106:14373–14378. [PubMed: 19597154]

Warner JR, Mitra G, Schwindinger WF, Studeny M, Fried HM. Saccharomyces cerevisiae coordinates accumulation of yeast ribosomal proteins by modulating mRNA splicing, translational initiation, and protein turnover. Mol Cell Biol. 1985; 5:1512–1521. [PubMed: 3897837]

Wu CH, Sahoo D, Arvanitis C, Bradon N, Dill DL, Felsher DW. Combined analysis of murine and human microarrays and ChIP analysis reveals genes associated with the ability of MYC to maintain tumorigenesis. PLoS Genet. 2008; 4:e1000090. [PubMed: 18535662]

Yadavilli S, Mayo LD, Higgins M, Lain S, Hegde V, Deutsch WA. Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain. DNA Repair (Amst). 2009; 8:1215–1224. [PubMed: 19656744]

Yu Y, Maggi LB Jr, Brady SN, Apicelli AJ, Dai MS, Lu H, Weber JD. Nucleophosmin is essential for ribosomal protein L5 nuclear export. Mol Cell Biol. 2006; 26:3798–3809. [PubMed: 16648475]

Yuan X, Zhou Y, Casanova E, Chai M, Kiss E, Grone HJ, Schutz G, Grummt I. Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. Mol Cell. 2005; 19:77–87. [PubMed: 15989966]

Zaitseva L, Cherepanov P, Leyens L, Wilson SJ, Rasaiyaah J, Fassati A. HIV-1 exploits importin 7 to maximize nuclear import of its DNA genome. Retrovirology. 2009; 6:11. [PubMed: 19193229]

Zeller KI, Haggerty TJ, Barrett JF, Guo Q, Wonsey DR, Dang CV. Characterization of nucleophosmin (B23) as a Myc target by scanning chromatin immunoprecipitation. J Biol Chem. 2001; 276:48285–48291. [PubMed: 11604407]

Zhai W, Comai L. Repression of RNA polymerase I transcription by the tumor suppressor p53. Mol Cell Biol. 2000; 20:5930–5938. [PubMed: 10913176]

Zhang Y, Lu H. Signaling to p53: ribosomal proteins find their way. Cancer Cell. 2009; 16:369–377. [PubMed: 19878869]

Zhang Y, Wang J, Yuan Y, Zhang W, Guan W, Wu Z, Jin C, Chen H, Zhang L, Yang X, He F. Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. Nucleic Acids Res. 2010; 38:6544–6554. [PubMed: 20542919]

Zhu Y, Poyurovsky MV, Li Y, Biderman L, Stahl J, Jacq X, Prives C. Ribosomal protein S7 is both a regulator and a substrate of MDM2. Mol Cell. 2009; 35:316–326. [PubMed: 19683495]

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev. 1998; 12:2424–2433. [PubMed: 9694806]

## Highlights

- c-Myc induces the transcription of *IPO7* and *XPO1*.
- Depletion of IPO7 results in p53 activation and subsequent growth inhibition.
- Depletion of IPO7 activates p53 through ribosomal stress.
- *IPO7* and *XPO1* are transcriptionally repressed by p53.

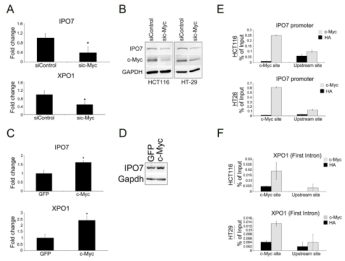

**Fig 1. IPO7 is a direct target of c-Myc**

(A) HCT116 cells were transfected with siRNA (20 nM) targeted against c-Myc (sic-Myc) or control siRNA (siControl). Cells were harvested 48 hours later, and relative levels of *IPO7* and *XPO1* mRNA were measured using RT-qPCR. Levels of *IPO7* (upper panel) and *XPO1* (lower panel) mRNA were normalized to *GAPDH* mRNA in the same sample. Results are presented as fold change relative to non-treated siControl cells, taken as 1.0. The mean of 4 independent experiments is presented; bars indicate SD (*=p<0.05).

(B) HCT116 and HT-29 cells were transfected and harvested as in (A), followed by SDS-PAGE and Western blot analysis for IPO7 and c-Myc proteins. GAPDH served as loading control.

(C) Mouse embryonic fibroblasts (MEF) were infected with recombinant retroviruses encoding either GFP or c-Myc. 72 hours later, cells were harvested and relative levels of *IPO7* and *XPO1* mRNA were measured by qRT-PCR and normalized for *GAPDH* mRNA. Results are presented as fold change relative to the levels in GFP infected cells, taken as 1.0. Bars indicate SD (n=3, *=p<0.05).

(D) MEF were infected as in (C). IPO7 protein levels were analyzed by Western blotting.

(E) HCT116 and HT-29 cells were subjected to chromatin immunoprecipitation (ChIP) analysis with c-Myc antibody or control HA antibody. Primers flanking the putative c-Myc binding site were employed to amplify that region by qPCR. Primers located 3Kb upstream to the transcription start site were used as negative control. ChIP values are presented as percentage of the input.

(F) ChIP was performed as in E, but with qPCR primers spanning the putative c-Myc site within the *XPO1* first intron.

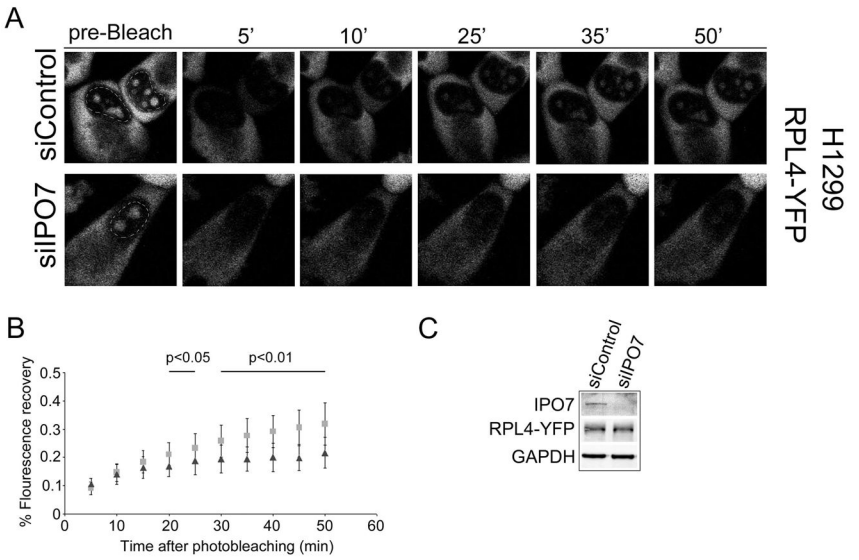


**Fig 2. Reduced nuclear import of RPL4 *in vivo* following IPO7 depletion**

(A) Nuclei of H1299 cells endogenously expressing YFP-tagged RPL4 were photobleached until less than 5% of the original signal remained detectable. Time lapse microscopy was employed to monitor nucleolar fluorescence signal recovery in cells transiently transfected with 20nM control siRNA or *IPO7* siRNA. Pictures were taken at 5 minute time intervals.

(B) The average fluorescence intensity within the nucleus of the bleached cells was measured. Each time point is expressed as the relative measured intensity divided by the pre-bleached signal. All values were corrected to the background signal. The average of 10 different cells is presented. Bars indicate SD.

(C) Western blot analysis of IPO7, RPL4-YFP and GAPDH as loading control, in cell cultures corresponding to (A).

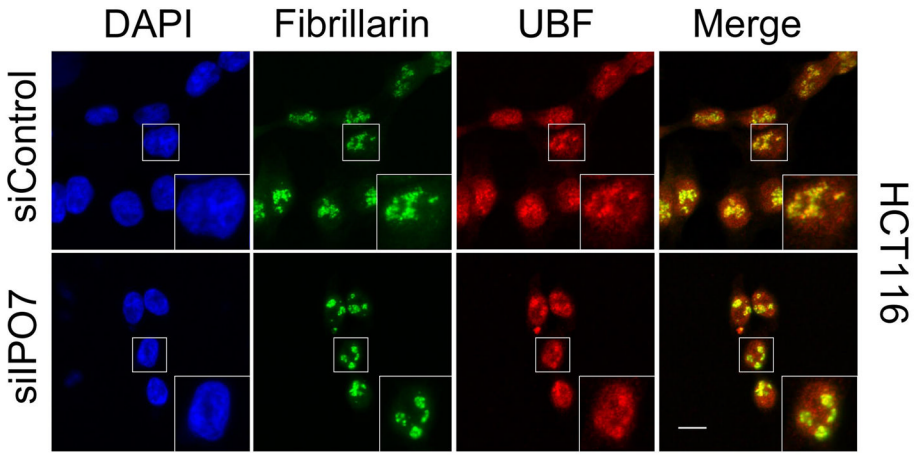


**Fig 3. IPO7 knockdown causes altered nucleolar morphology**

HCT116 cells were transfected with control or IPO7 siRNA (20 nM). 96 hours later, cells were fixed and immunostained with antibodies against fibrillarin (Green) and UBF (Red). Nuclei were counterstained with DAPI (Blue). Insets depict a higher magnification of the cell indicated by the small square. Scale bar (bottom right panel) = 10 μm.

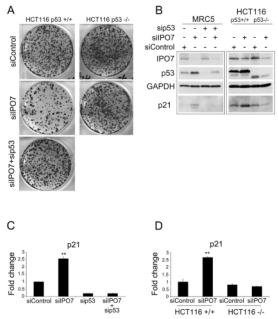


**Fig 4. IPO7 knockdown triggers p53 activation and growth inhibition**

(A) HCT116 cells (wt and p53-null) were transiently transfected with IPO7 siRNA (20nM), control siRNA (20nM) or a combination of both siRNAs (20nM each). 96 hours later cells were trypsinized and re-plated at $5\times10^3$ cells/well in a 6 well dish. After an additional 7 days, colonies were stained with crystal violet.

(B) MRC5 and HCT116 cells were transiently transfected with the indicated siRNA oligonucleotides (20nM). 96 hours later cells were harvested and subjected to Western blot analysis with antibodies against IPO7, p53, p21 and GAPDH as a loading control.

(C, D) qRT-PCR analysis of *p21* mRNA levels in MRC5 (left) and wild type (p53+/+) and p53-deficient (p53−/−) HCT116 cells (right) 96 hours after transient transfection with the indicated combinations of siRNA oligonucleotides (20nM each). *p21* was normalized to *GAPDH* mRNA. Results are presented as fold change relative to non-treated siControl cells, taken as 1.0. Bars indicate SD; n=3 (** =p<0.01).

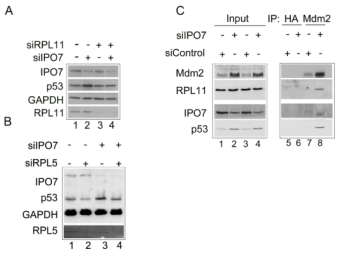


**Fig 5. Activation of p53 following IPO7 knockdown is mediated through RPL11 and RPL5**

(A) HCT116 cells were transiently transfected with the indicated combinations of siRNA targeting IPO7 or RPL11 (20nM each). 72 hours later cells were harvested and protein extracts subjected to Western blot analysis with the indicated antibodies. GAPDH served as a loading control.

(B) HCT116 were transiently transfected as in (A) except that cells were transfected with RPL5 siRNA.

(C) RPL11 associates with Mdm2 in cells depleted of IPO7. HCT116 cells were transiently transfected with siIPO7 or siControl (20nM) and harvested 72 hours later. Proteins were extracted in NP-40 lysis buffer, and incubated with anti-Mdm2 antibodies or anti-HA tag antibodies as negative control. Immunoprecipitated proteins (IP, right panel) as well as 5% of each total cell extract (Input, left panel) were subjected to Western blot analysis with the indicated antibodies.

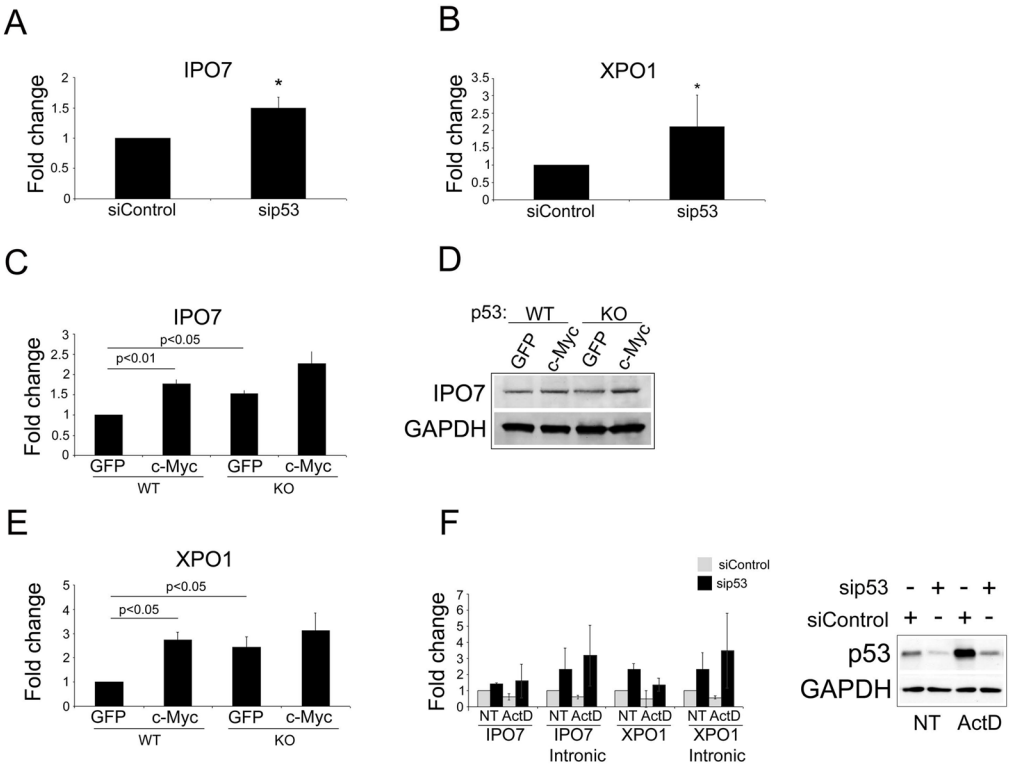


**Fig 6. *IPO7* gene transcription is repressed by p53**

(A,B) MRC5 cells were transfected with p53 siRNA (sip53) or control siRNA. *IPO7* (A) and *XPO1* (B) mRNA levels were measured using qRT-PCR and normalized for the corresponding *GAPDH* mRNA. Results are presented as fold change relative to non-treated siControl cells, taken as 1.0. Error bars indicate SD (n=3). (* = $p<0.05$).

(C) Wild type (WT) and p53 knockout (KO) mouse embryonic fibroblasts (MEF) were infected with recombinant retroviruses expressing c-Myc or GFP as control. 72 hours post infection, cells were harvested and relative levels of *IPO7* mRNA determined as in (A). Results are presented as fold change relative to the levels in the GFP infected cells, taken as 1.0. Error bars represent standard deviation (n=3).

(D) Western blot analysis of IPO7 protein levels in cultures treated as in (C). GAPDH served as loading control.

(E) *XPO1* mRNA was quantified as in (C). Error bars represent standard deviation (n=3).

(F) MRC5 cells were transfected with p53 siRNA (sip53) or control siRNA. 72 hours later, cells were treated with solvent (ethanol) only (NT) or 5nM actinomycin D (ActD) in ethanol for an additional 24 hours. Levels of mature *IPO7* and *XPO1* mRNA, as well as of the corresponding heterogeneous nuclear RNA, representing primary transcripts and identified through the use of intronic primers (Intronic), were measured by qRT-PCR and normalized for *GAPDH* mRNA. Results are presented as fold change relative to non-treated siControl cells, taken as 1.0. Error bars indicate SD (n=3). Right panel: Western blot analysis of p53 protein levels in the same samples.

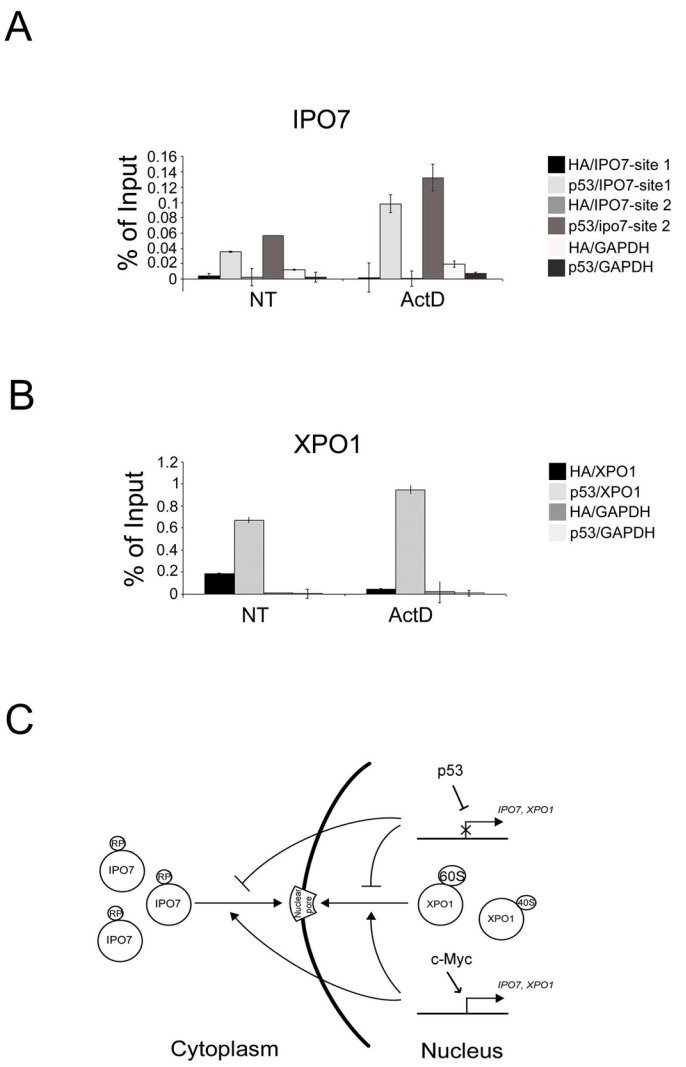


**Figure 7. p53 associates with the *IPO7* and *XPO1* genes**

(A) MRC5 cells were treated for 16 hours with 5nM Actinomycin D (ActD) or solvent only (NT), and then subjected to chromatin immunoprecipitation (ChIP) analysis with p53-specific antibody (p53) or control HA antibody. Primers flanking the putative p53 binding sites were employed to amplify the corresponding DNA regions by RT-qPCR. For the *IPO7* gene, two different sites were amplified, termed site 1 and site 2, in the *IPO7* promoter and first intron, respectively.

Primers derived from the *GAPDH* gene served as negative control. ChIP values are presented as percentage of input. Bars represent standard deviation.

(B) Same as in (A), except that the analysis was done with XPO1 primers.

(C) Schematic model depicting the opposing regulation of the nuclear import/export machinery by c-Myc and p53. Positive and negative regulatory interactions are indicated. RP = ribosomal proteins; 60S and 40S refer to the corresponding ribosomal subunits.