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Review

Negative auto-regulators trap p53 in their web

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The transcriptional factor p53 activates the expression of a myriad of target genes involving a complicated signalling network, resulting in various cellular outcomes, such as growth arrest, senescence, apoptosis, and metabolic changes, and leading to consequent suppression of tumour growth and progression. Because of the profoundly adverse effect of p53 on growth and proliferation of cancer cells, several feedback mechanisms have been employed by the cells to constrain p53 activity. Two major antagonists MDM2 and MDMX (the long forms) are transcriptionally induced by p53, but in return block p53 activity, forming a negative feedback circuit and rendering chemoresistance of several cancer cells. However, they are not alone, as cancer cells also employ other proteins encoded by p53 target genes to inhibit p53 activity at transcriptional, translational, and posttranslational levels. This essay is thus composed to review a recent progress in understanding the mechanisms for how cancer cells hijack the p53 autoregulation by these proteins for their growth advantage and to discuss the clinical implications of these autoregulatory loops.

Keywords: p53, MDM2, NGFR, transcription, feedback loop, chemoresistance

Introduction

The tumour suppressor p53 plays an essentially important role in guarding genome and defeating tumour development and progression. Since it was discovered in 1979, the *TP53* gene had been regarded as an oncogene (Lane and Crawford, 1979; Linzer and Levine, 1979) till 1989 when wild-type p53 was found to actually function as a tumour suppressor (Baker et al., 1989; Finlay et al., 1989). The wild-type p53 executes its tumour suppression functions mostly by transcriptionally activating a myriad of target genes, which encode proteins responsible for cell cycle arrest, DNA repair, senescence, apoptosis, autophagy, ferroptosis, and metabolic changes, respectively (Riley et al., 2008; Vousden and Ryan, 2009; Jiang et al., 2015). Also, p53 can exert transcription-independent activity to trigger mitochondrial outer membrane permeabilization and apoptosis (Chipuk et al., 2004; Leu et al., 2004). Because of the detrimental effects of p53 on cell proliferation and growth, higher eukaryotic vertebrate organisms including humans have evolved an elegant negative feedback autoregulation to control p53. This regulation involves two oncoproteins called MDM2 (also called HDM2 for its human analogue) and MDMX (also called MDM4).

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MDM2 is a RING finger-containing protein, encoded by a p53 target gene that is often amplified or overexpressed in multiple human cancers, and has been deemed to be the most important repressor of p53, hence constituting a critical negative feedback loop (Momand et al., 1992; Oliner et al., 1992; Wu et al., 1993). MDM2 utilizes several mechanisms to inhibit p53 activity. First, it possesses an intrinsic E3 ligase to mediate p53 polyubiquitination and proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997; Fuchs et al., 1998). Also, it targets p53 for monoubiquitination and nuclear export, thus preventing p53 from binding to its target promoters in the nucleus (Li et al., 2003). Additionally, it inhibits p53 transcriptional activity by directly associating with and concealing the transactivation (TA) domain of p53 (Oliner et al., 1993). Lastly, MDM2 can suppress *TP53* mRNA translation by promoting RPL26 degradation and dissociating the RPL26–p53 mRNA interaction (Ofir-Rosenfeld et al., 2008). The central role of MDM2 in the inactivation of p53 is also demonstrated by mouse genetic studies, showing that the early embryonic lethality caused by knocking out the *Mdm2* gene is completely rescued by further deleting *Tp53* (Jones et al., 1995; Montes de Oca Luna et al., 1995). Thus, MDM2 serves as a prime feedback antagonist of p53, but it also often works with its partner MDMX to inactivate p53.

MDMX, an MDM2 homologue without any apparent intrinsic E3 ligase activity, has been found to repress p53 activity by partnering with MDM2 (Shvarts et al., 1996; Wade et al., 2010).

MDMX associates with MDM2 through their C-terminal RING domains and boosts the E3 ligase activity of the latter towards p53 (Tanimura et al., 1999; Badciong and Haas, 2002). Also, MDMX can suppress p53 transcriptional activity by directly interacting with the N-terminal TA domain of this transcriptional factor (Shvarts et al., 1996). Its essential role in suppressing p53 has also been demonstrated by animal studies, as the embryonic lethality of *Mdmx*-null mice can be rescued by further deleting *Tp53* as well (Parant et al., 2001; Finch et al., 2002). The partnership between Mdm2 and Mdmx appears to be more essential for animal embryogenesis, as genetic dissociation of their binding causes a lethal phenotype that can be rescued by deleting *Tp53* (Huang et al., 2011; Pant et al., 2011). However, conditional knockout studies have shown that Mdm2 and Mdmx can play independent roles in organogenesis, as Mdmx is important only for the inactivation of p53 in certain organs or tissues, while Mdm2 is essential for p53 inactivation in all organs and tissues in mice (Boesten et al., 2006; Grier et al., 2006; Xiong et al., 2006, 2007; Maetens et al., 2007; Abbas et al., 2010; Pant et al., 2013; Zhang et al., 2014b, c). Altogether, these studies demonstrate that MDMX also plays a crucial role in suppressing p53 activity either by working with MDM2 or independently.

This MDM2–MDMX–p53 loop is critically important for normal growing or somatic cells to monitor p53 level and activity, but it is also subjected to multitude regulations at different levels in response to various stressors or signals to provoke the anti-tumour functions of p53, as briefed in the following section. The field has witnessed a tremendous progress in understanding the mechanisms for and the biological importance of the regulations of this loop. This review focuses on the recent progress about how cancer cells utilize this loop to inhibit p53 activity by recruiting other oncoprotein helpers for their growth advantage, while readers are referred to other review articles on the role of MDM2 in organ formation, development, DNA repair, stem cell regulation, metabolism, oncogenesis, and drug discovery in this MDM2 special issue.

Regulations of the MDM2–p53 loop

Because p53 is tightly controlled by MDM2 and MDMX in normal cells, breaking up this control is necessary for the cells to promptly turn on p53 and effectively utilize its remarkable anti-oncogenic power to maintain their cancer cell-free environment. Indeed, there are myriad ways to turn on p53 (Kruse and Gu, 2009). For example, the repairable DNA damage signalling can trigger the ATM/ATR–Chk2/Chk1 kinase cascade that activates p53 by phosphorylating MDM2 and MDMX and blocking their feedback regulations on this protein, though this cascade can directly activate p53 by phosphorylating this protein as well (Shieh et al., 1997, 2000; Canman et al., 1998; Tibbetts et al., 1999; Hirao et al., 2000; Chen et al., 2005; Jin et al., 2006; Cheng et al., 2009; Wang et al., 2009). Also, oncogenic stress can induce the expression of another tumour suppressor P14ARF, which activates p53 by binding to MDM2 and inhibiting its E3 ligase activity directly (Stott et al., 1998; Zhang et al., 1998). Interestingly, the molecular basis for ARF-induced p53 activation is not completely evolutionary conserved, because P19Arf, the mouse homologue of P14ARF, was shown to interact with both MDM2 and p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Honda and Yasuda, 1999). Furthermore, disturbing ribosomal biogenesis by different chemical agents or under different stress conditions, such as hypoxia, metabolic, or genetic alterations, leads to p53 activation by inducing the interactions of various ribosomal proteins with MDM2, consequently suppressing the activity of the latter towards p53 (Zhang and Lu, 2009; Zhou et al., 2012, 2015). Cells hire these mechanisms, though not complete, to deploy p53 for the ultimate purpose of maintaining their balanced homeostasis and protecting themselves from undergoing transformation and eventual tumorigenesis whenever confronting stressful and potential cancer-causing environments.

However, this MDM2–MDMX–p53 loop is often hijacked by cancer cells for their growth benefits. For instance, several types of cancers, such as sarcoma, melanoma, breast cancer, leukaemia, and lymphoma, express high levels of MDM2 and/or MDMX that inactivate p53 and its downstream pathways with much lower level of *TP53* mutation (Reifenberger et al., 1993; Wasylishen and Lozano, 2016). Also, several other oncogenic proteins, such as TSG101 (Li et al., 2001), YY1 (Sui et al., 2004), and Gankyrin (Higashitsuji et al., 2005), have previously been shown to boost MDM2-mediated p53 proteolysis by either stabilizing MDM2 or facilitating MDM2–p53 interaction. More recently and interestingly, a number of proteins encoded by p53-responsive target genes have been uncovered to inactivate p53 by either directly binding to it or indirectly through collaboration with MDM2 or MDMX, forming a multilayer autoregulatory feedback network, as further described in the following section.

Trapping p53 in the negative autoregulatory web

MDM2 was the first negative feedback regulator of p53 discovered in early 1990 s (Momand et al., 1992; Oliner et al., 1992, 1993; Wu et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997). Since then, a growing number of p53 target genes have been identified that control p53 level and activity in a feedback fashion as well, forming an autoregulatory feedback network (Figure 1). As mentioned above, one critical partner of MDM2 is MDMX. Interestingly, the expression of the long form of MDMX, MDMX-L, can also be induced by p53 through an alternative promoter within the first intron of the *MDMX* gene in a cell line-dependent fashion (Li et al., 2010; Phillips et al., 2010). MDMX-L was shown to work cooperatively with MDM2 to target p53 for degradation (Phillips et al., 2010), although this observation remains to be validated *in vivo*.

In addition to MDMX, there are several p53-responsive target genes that encode proteins capable of promoting MDM2-mediated ubiquitin-dependent p53 degradation, including leukaemia inhibitory factor (*LIF*) (Hu et al., 2007; Yu et al., 2014), xeroderma pigmentosum group C (*XPC*) (Adimoolam and Ford, 2002; Krzeszinski et al., 2014), and Cockayne syndrome B (*CSB*)

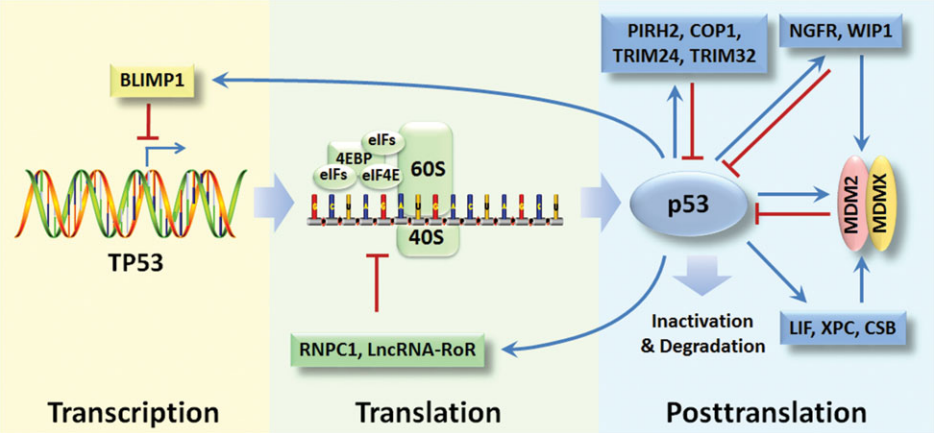

Figure 1 The negative autoregulatory network of p53. As a transcriptional factor, the tumour suppressor p53 induces the expression of a large number of genes, whereas some of these target genes inactivate p53, thus composing negative feedback regulatory loops. For instance, the transcription repressor BLIMP1 regulates p53 at the transcriptional layer; the RNA-binding protein RNPC1 and LncRNA-RoR suppress p53 at the translational layer; and the others inhibit p53 through direct interaction and/or posttranslational modifications.

(Latini et al., 2011). Interestingly, *Lif* was initially identified as a p53 transcriptional target in the mouse uteri to maintain maternal reproduction (Hu et al., 2007). But later on, it was found to be able to upregulate MDM2 expression through activation of the STAT3 signalling, resulting in increased p53 degradation (Yu et al., 2014). XPC and CSB are encoded by genes that are often mutated in the rare genetic diseases, xeroderma pigmentosum and Cockayne syndrome, respectively (Troelstra et al., 1992; Sands et al., 1995). The two proteins were independently found to enhance MDM2-dependent p53 degradation by associating with the MDM2/p53 complex (Adimoolam and Ford, 2002; Latini et al., 2011; Krzeszinski et al., 2014).

Besides these MDM2 helpers, earlier studies revealed several non-MDM2 RING finger E3 ubiquitin ligases that can also regulate p53 level and activity in a feedback manner, such as PIRH2 and COP1 (Leng et al., 2003; Dornan et al., 2004). They were independently shown to bind to p53 and promote its ubiquitin-mediated degradation without engaging MDM2. In addition to regulating p53 stability, PIRH2 was shown to suppress p53 transcriptional activity without needing its RING finger domain, but by interfering with the DNA-binding capacity of p53 (Leng et al., 2003). However, the intact RING finger domain appeared to be required for COP1-mediated inactivation of p53 (Dornan et al., 2004). Interestingly, since the interaction between COP1 and p53 was only found in cancer cells, and the *Cop1*-deficient mice did not show any increase in p53 level and activity (Migliorini et al., 2011), the regulation of p53 by this E3 ligase might only be utilized by cancer cells.

Several members of the tripartite motif (TRIM) family have also been found to target p53 for ubiquitination and degradation via a feedback fashion. For instance, Trim24 was identified as a p53-binding partner in mouse embryonic stem cells and shown to promote p53 degradation in organisms from Drosophila to human (Allton et al., 2009; Jain et al., 2014). Also, TRIM32 was shown to drive oncogenic transformation and tumorigenesis by prompting p53 protein turnover through a proteasomal pathway (Liu et al., 2014). Interestingly, *TRIM32* was also found to be transcriptionally induced by and to promote the degradation of TAp73, a p53 homologue, in the neural progenitor cells (Gonzalez-Cano et al., 2013).

Another example is the wild-type p53-induced phosphatase 1 (WIP1) encoded by the p53 target gene *PPM1D*. Intriguingly, WIP1 inactivates p53 through multiple posttranslational mechanisms (Fiscella et al., 1997). First, WIP1 mediates dephosphorylation and subsequent inactivation of p38 MAPK, consequently attenuating UV-induced p53 phosphorylation at Ser33 and Ser46 that are catalysed by active p38 MAPK (Takekawa et al., 2000; Bulavin et al., 2002). Second, WIP1 directly contacts p53 and dephosphorylates it at serine 15, which strikingly compromises p53 transcriptional activity (Lu et al., 2005). At last, WIP1 interacts with and dephosphorylates MDM2 at serine 395, and thus enhances MDM2 stability and accessibility for p53, resulting in augmented p53 ubiquitination and degradation (Lu et al., 2007). Through these different mechanisms, WIP1 controls p53 level and activity.

Making the already complex regulations of p53 more complicated are the additional regulations of this tumour suppressor at the transcriptional and translational levels by its other target genes. One of these examples is the zinc-finger protein BLIMP1, which was originally identified as a transcriptional repressor of the *β-IFN* gene by specifically binding to the PRDI (positive regulatory domain I element) of its promoter (Keller and Maniatis, 1991), but later was found to inhibit p53 gene transcription by directly associating with the p53 promoter region close to its transcription start site (Yan et al., 2007). Another example is RNPC1, an RNA-binding protein whose RNA is transcriptionally induced by the p53 family (Zhang et al., 2011). RNPC1 associates with the 5' and 3' untranslated regions of p53 mRNA and prevents cap-binding protein eIF4E from binding to it, thus resulting in the inhibition of p53 protein synthesis. Consistent

with these results, depletion of *Rnpc1* in mouse embryonic fibroblasts elevated p53 expression, while hyper-expression of RNPC1 in dog lymphomas led to the reduction of p53 expression (Zhang et al., 2011). Recently, a long non-coding RNA gene was also found to be involved in the autoregulation of p53, as human lncRNA-RoR is transcriptionally induced by p53 to suppress the translation of p53 mRNA by directly binding to the heterogeneous nuclear ribonucleoprotein I (hnRNP I) (Zhang et al., 2013). However, this is not the end of the list, as our latest finding revealed nerve growth factor receptor (NGFR) as a new autoregulatory suppressor of p53 (Zhou et al., 2016).

NGFR, also known as p75NTR or CD271, was originally identified as a transmembrane pan-receptor for all mature neurotrophins, including nerve growth factor (NGF), brain-derived neutrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) with low affinity, as well as their precursors, pro-neurotrophins, with high affinity (Barker, 2004). Depending on the type of normal or cancer cells, cell differentiation status, (pro-)neurotrophin availability, co-receptor engagement, and the intracellular adaptor molecules, NGFR has been shown to play diverged and sometimes contradictory roles either as an oncogenic protein or as a tumour suppressor (Barker, 2004; Molloy et al., 2011), but these cancer-related roles are only associated with its transmembrane receptor functions. No study has ever paid attention to its possible nuclear functions. In a recent attempt to identify novel p53 targets, we revealed the gene encoding NGFR as a new p53-responsive target gene by performing a microarray analysis of gene expression profiles in both wild-type and p53-deficient HCT116 colon cancer cells using the p53-inducing small molecule, Inauhzin (Liao et al., 2012; Zhang et al., 2012). Biologically, depleting NGFR triggered apoptosis and growth arrest of various cancer cells, such as melanoma, neuroblastoma, lung, liver, and colon cancer, and suppressed the growth of xenograft tumours derived from human lung cancer cells, demonstrating that NGFR plays an oncogenic role in these cancer cells (Zhou et al., 2016). Remarkably, knockdown of *NGFR* induced p53 level and activity, consequently activating its downstream pathway, such as upregulating the expression of p53 target genes, *p21*, *PUMA*, *BAX*, *BTG2*, and so on (Zhou et al., 2016). This result suggests that NGFR might play a role in the suppression of p53 function. Indeed, this is the case, as NGFR can suppress p53 activity via two distinct mechanisms: (i) its N-terminal extracellular domain interacts with the C-terminus of MDM2 in the nucleus, as confirmed by both confocal imaging and biochemical fractionation (Zhou et al., 2016), and this interaction leads to the enhancement of MDM2-mediated ubiquitination and degradation of p53; (ii) NGFR via its extracellular N-terminal domain can also disable the transcriptional activity of p53 independently of MDM2, by directly binding to the central DNA-binding domain of p53 and preventing the latter from binding to its target promoters (Zhou et al., 2016). Our study not only demonstrates NGFR as another negative feedback auto-regulator of p53, but also uncovers its new ligand-independent nuclear function as an oncoprotein to promote cancer cell proliferation and growth by inactivating p53, though it possesses p53-independent oncogenic functions as well (Molloy et al., 2011).

Taken together, these findings strongly demonstrate that wild-type p53-harbouring cancer cells utilize various tricks to inactivate p53 in response to p53-activating agents, including those used for chemotherapy; but all of these tricks display one similar feature—the negative feedback, i.e. to activate different downstream target genes of p53, which encode proteins that can either enhance MDM2-dependent p53 degradation or inhibit p53 expression at transcriptional and translational levels and disable p53 transcriptional activity independent of MDM2 (Figure 1). These cancer cells deploy these remarkable tactics for their growth advantage, which might also account for their drug resistance as further discussed below.

Negative p53 autoregulation responsible for chemoresistance

One obvious question is why p53 activates the expression of so many target genes for its own hardship in cancer cells. This should not be the initial motive of p53 as a tumour suppressor. Instead, it is likely that cancer cells might take advantage of the p53 autoregulatory feedbacks as described above for their survival through multiple rounds of chemotherapy. Several lines of evidence support this speculation. Classical examples are MDM2 and MDMX that have been shown as drug-resistant factors in several types of cancers as reviewed by others (Wade et al., 2013; Khoo et al., 2014; Zhang et al., 2014a). Another example is LIF, as ectopic LIF was found to significantly attenuate cell apoptosis induced by Fluorouracil (5-FU), Etoposide, or Adriamycin in several wild-type p53-containing, but not p53-null, human colorectal cancer cell lines (Yu et al., 2014). Conversely, knockdown of endogenous *LIF* made p53-positive cells more sensitive to 5-FU treatment in both cell-based survival assays and xenograft tumour models. In line with these results, *LIF* expression was significantly higher in human colorectal tumours than in their adjacent non-tumour tissues, and the high expression of *LIF* was significantly associated with poor prognosis in cancer patients (Yu et al., 2014). Although the *TP53* mutation status needs to be determined in these tumour samples, this study suggests that the high level of *LIF* plays a possible role in the development of chemoresistance of colorectal cancers to 5-FU treatment.

Aforementioned NGFR might also be responsible for chemoresistance. When *NGFR* was knocked down, p53-positive cancer cells became more sensitive to chemotherapeutic agent Cisplatin, as the IC50 of Cisplatin was reduced by 1.6- and 2.2-fold in HCT116$^{p53+/+}$ and H460 cells, respectively. However, less or no effect was observed in HCT116$^{p53-/-}$ or p53-null H1299 cells in the same set of experiments (Zhou et al., 2016). Interestingly, higher levels of *NGFR* were detected in tumours with wild-type *TP53*, suggesting that tumour cells with wild-type p53 utilize this negative regulator to inhibit p53 functions for their better survival.

In addition to the two examples above, WIP1 and COP1 have been implied to render chemotherapeutic resistance to tumour cells as well. A recent study showed that treatment of breast

cancer MCF7 cells that contain wild-type p53 and highly amplified *PPM1D*, the WIP1-encoding gene, with GSK2830371, a specific inhibitor for WIP1, significantly sensitized the cells to genotoxic drugs, including Doxorubicin and Etoposide, as determined by cell proliferation and p53 pathway activation (Pechackova et al., 2016). In addition, GSK2830371 was able to enhance the inhibitory effect of Nutlin-3, a specific MDM2 inhibitor (Pechackova et al., 2016). Consistent with this study, several other studies also reported that inhibition of WIP1 by GSK2830371 could potentiate the anti-cancer effect of Nutlin-3 in osteosarcoma, neuroblastoma (Esfandiari et al., 2016), and colon carcinoma cells (Sriraman et al., 2016). Furthermore, re-introduction of miR-214 into breast cancer cells, which is often downregulated in breast cancer cells and clinical breast cancer samples, could directly reduce COP1 expression, and consequently sensitized the cancer cells to Doxorubicin treatment in a p53-dependent manner (Zhang et al., 2016). These studies also support the idea that the high expression of p53 negative feedback regulators in response to chemotherapy might be a mechanism for those cancer cells that harbour wild-type p53 to develop chemoresistance during this therapy.

In summary, when wild-type p53-sustaining tumour cells are subjected to chemotherapy, they will employ some of those aforementioned p53 target genes to destroy p53 functions in a feedback fashion, and thus confer their resistance to the chemotherapy.

Questions and prospects

As discussed above, the negative autoregulation of p53 by the proteins encoded by its target genes represents a critical mechanism that is often hijacked by tumour cells to restrain p53 activity in favour of their development, progression, and/or drug resistance. However, to better translate this complex regulation into its clinical significance, such as its correlation with the clinical progression and chemoresistance of human cancers and its potential as a target for cancer intervention in clinical settings, there are still several important issues that need to be addressed. First, it is necessary to establish mouse tumour model systems to determine the biological roles of the aforementioned negative feedback regulators of p53 in tumour progression and drug resistance. By doing so, we will obtain pre-clinical evidence to support the development of mechanism-driven strategies to surmount the p53 feedback regulation-caused tumour progression and drug resistance. Second, it would be of great interest to determine whether the expression patterns of these genes, such as amplification or overexpression, are highly associated with the progression and drug resistance of a broad spectrum of human cancers. This comprehensive analysis will provide informative data as to whether alterations of these genes are well correlated with or responsible for tumorigenesis, progression, and drug resistance. It is also important to determine whether the expression of these p53 negative regulators is well correlated with the wild-type status of *TP53*. This information would offer molecular insight into the progression and/or the relapse post therapy of human cancers that sustain wild-type p53. Finally, once their clinical relevance is validated, these p53 negative regulators could serve as molecule targets for future development of anti-cancer therapy for cancers that contain wild-type p53. Cocktail treatment by combining the agent(s) that targets one or more of these p53 protein suppressors with a p53-activating agent, such as an MDM2 inhibitor, should certainly achieve a better efficacy on human cancers that harbour wild-type p53. Optimistically, the day to accomplish this goal would not be that far away as long as we continue dissecting the complex autoregulatory web of p53 (Figure 1).

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