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The p53 tumor suppressor gene mediates a major tumor suppression pathway in mammalian cells that is frequently altered in human cancers, including breast cancer. p53 protein is kept at low level during normal cell growth by its short half-life and is stabilized following oncogenic stimulation and DNA damage. Growing evidence has identified oncoprotein MDM2 (mouse double minute) as a key negative regulator and tumor suppressor ARF (alternative reading frame) as a key positive regulator of p53 protein stability. Two findings were made over the past year that extended our understanding into the p53 control by MDM2 and ARF. First, we found that human tumor suppressor ARF impedes S-phase progression independent of p53. Second, we discovered that p53 protein contains two separate nuclear export signals (NES) that functional collaborate to mediate an MDM2-independent nuclear export.

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INTRODUCTION

The p53 tumor suppressor gene mediates a major tumor suppression pathway in mammalian cells that is frequently altered in human cancers (Levine, 1997; Prives, 1998; Sherr, 1998). p53 protein is kept at low level during normal cell growth by its short half-life and is stabilized following oncogenic stimulation and DNA damage. Growing evidence has identified MDM2 as a key regulator of p53 protein stability (Haupt et al., 1997; Kubbutat et al., 1997). Blocking p53 nuclear exporting leads to p53 stabilization (Roth et al., 1998; Freeman et al., 1998), indicating that p53 degradation occurs in the cytoplasm and suggesting p53 nuclear export as a major regulatory event in MDM2-mediated p53 degradation. The tumor suppressor ARF stabilizes p53 by blocking the nuclear export of both p53 and MDM2 (Tao and Levine, 1999; Zhang and Xiong, 1999). Human tumor-derived mutations in the *ARF-INK4a* locus can selectively target the *ARF* gene and impair ARF function in blocking p53 nuclear export, underscoring the importance of nuclear export in regulating p53 stability (Zhang and Xiong, 1999). The mechanism governing p53 nuclear export has been under intense investigation, but remains unclear and controversial at present.

The mammalian ARF-INK4a locus uniquely encodes two cell cycle inhibitors, $p16^{INK4a}$ (Serrano et al., 1993) and ARF (Quelle et al., 1995), by using separate promoters and alternative reading frames, that both function in tumor suppression. Mice with homozygous deletion of both p16 and ARF (Serrano et al., 1996), or selective deletion of ARF, sparing p16 (Kamijo et al., 1997), develop spontaneous tumors at an early age. $p16^{INK4a}$ encodes a specific inhibitor of CDK4/CDK6 and maintains the retinoblastoma protein in its growth suppressive state through inhibition of its phosphorylation(Serrano et al., 1993), while ARF binds to and inactivates MDM2, thereby stabilizing p53 (Pomerantz et al., 1998; Zhang et al., 1998; Stott et al., 1998; Kamijo et al., 1998). MDM2 binds to and abrogates p53 function by either targeting p53 for degradation in the cytoplasm or by repressing p53's transcriptional activity in the nucleus [reviewed in (Levine, 1997; Prives, 1998)]. ARF inhibits MDM2 at least in part by preventing MDM2-dependent nuclear export and cytoplasmic degradation of p53 (Zhang and Xiong, 1999; Tao and Levine, 1999), through forming ARF-MDM2-p53 ternary complex (Zhang and Xiong, 1999). Transcription of the ARF gene is induced by several proliferative oncogenes (de Stanchina et al., 1998; Zindy et al., 1998; Bates et al., 1998; Palmero et al., 1998), and loss of ARF abrogates the stabilization and induction of p53 levels by these oncogenic signals resulting in greatly diminished apoptosis (de Stanchina et al., 1998; Zindy et al., 1998). Taken together, these findings suggest that ARF may mediate a p53-dependent G1 checkpoint control or apoptosis in response to hyperproliferative oncogenic stimulus (Sherr, 1998).

To date, ARF activity has been primarily ascribed to its ability to stabilize and activate p53, resulting in a G1 cell cycle arrest. Genetic evidence supporting this notion come from the observations that tumors arising from ARF/INK4a-deficient mice lack p53 mutation/deletion (Chin et al., 1997), that ectopic expression of ARF inhibits S-phase entry in wild type MEFs, but not in several established fibroblast cell lines lacking p53 function (Kamijo et al., 1997), and that ARF inhibits cellular

transformation only in the presence of functional p53 (Pomerantz et al., 1998). In vivo genetic analysis has, however, suggested that loss of ARF function is not equivalent to the loss of p53. ARF-p53 double null B cells are more resistant to Myc-induced apoptosis and proliferate more rapidly than cells lacking either ARF or p53 (Eischen et al., 1999). Furthermore, a p53-independent function of ARF has been recently suggested in promoting cellular senescence and suppressing immortalization. Through expression of excisable antisense RNA constructs, it was shown that selective inhibition of either p16 or ARF expression results in extended lifespan of MEFs and that re-expression of either gene suppress the immortal phenotype. In this context, p16-induced growth arrest depends exclusively on a functional Rb pathway. On the other hand, growth arrest by restoration of ARF activity, in the context of cells that have bypassed senescence by loss of ARF, can only be canceled by simultaneous inactivation of both p53 and Rb activity (Carnero et al., 2000).

Two findings were made over the past year that extended out understanding into the p53 control by MDM2 and ARF. First, we found that human tumor suppressor ARF impedes S-phase progression independent of p53. Second, we discovered that p53 protein contains two separate nuclear export signals (NES) that functional collaborate to mediate an MDM2-independent nuclear export.

KEY RESEARCH ACCOMPLISHMENT AND CONCLUSIONS

Human Tumor Suppressor ARF Impedes S-phase Progression Independent of p53

The majority of ARF's activity to date is ascribed to its ability to activate p53 resulting in a G1 cell cycle arrest or apoptosis. We show here that overexpression of ARF impedes DNA synthesis resulting in accumulation of an S-phase cell population independent of p53. Hence, the biological consequence of ARF induction varies dependent on cellular p53 status: inducing predominantly a G1 arrest or apoptosis in p53-positive cells or causing S-phase retardation when p53 function is comprised. (Yarbrough et al. submitted).

Two separate nuclear export signals collaborate to mediate MDM2-independent p53 nuclear export

The level of p53 tumor suppressor protein is normally kept low in cells and rises in response to physiological stress through post-translational regulation that leads to p53 stabilization. MDM2 oncoprotein binds to and targets p53 for degradation in the cytoplasm. Both MDM2 and p53 shuttle between the nucleus and the cytoplasm via intrinsic nuclear localization signal (NLS) and nuclear exporting signal (NES) sequences. We show here that mutations of several hydrophobic amino acid residues in the N-terminal region of p53, but not a mutation in the central sequence-specific DNA binding domain, significantly reduced p53 nuclear export, conferring p53 resistance to MDM2-mediated degradation. Endogenous $p53^{L22Q/W23S}$ protein produced by a knock-in strategy is completely blocked from nuclear export and accumulates to very high levels in the nucleus. We provide more conclusive evidence demonstrating an MDM2-independent p53 nuclear export. We further demonstrated that the N-terminal domain of p53 contains an autonomous NES whose activity is independent of MDM2 and can be abolished by the mutation at conserved hydrophobic residues. Attachment of the N-terminal NES and C-terminal NES to a reporter GFP resulted in a more active nuclear export than the fusion with either NES alone. Our results suggest that p53 nuclear export is mediated by and dependent on a functional collaboration between two geographically separated NESs. The N-terminal NES is surrounded by several serine residues that are phosphorylated following DNA damage, raising the possibility that p53 activating kinases may stabilize p53 through impeding the activity of NES. (Zhang et al., submitted).

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APPENDICES

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