

FMN2 is a novel regulator of the cyclin-dependent kinase inhibitor p21

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Abbreviations: ARF, alternative reading frame; FMN2, formin-2; NFκB, nuclear factor kappaB; CDK, cyclin-dependent kinase; ROCK, Rho-associated, coiled-coil containing protein kinase; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein; ATR, ataxia telangiectasia and Rad3 related; ATM, ataxia telangiectasia mutated; UV, ultraviolet; Chk1, checkpoint kinase 1

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We have identified the human FMN2 gene as a novel target regulated by induction of p14ARF and by multiple other stress responses, including DNA damage and hypoxia, which have in common activation of cell cycle arrest. We showed that increased expression of the FMN2 gene following p14ARF induction is caused, at the transcriptional level, by relief of repression by RelA and E2F1, which, under non-induced conditions, bind the FMN2 promoter. Increased FMN2 protein levels promote cell cycle arrest by inhibiting the degradation of p21, and our data show that control of p21 stability is a key part of the mechanism that regulates p21 induction. Consistent with this model, we have shown that transient expression of exogenous FMN2 protein alone is sufficient to increase p21 protein levels in cells, without altering p21 mRNA levels. Here, we provide additional evidence for the role of the N terminus of FMN2 as being the important domain required for p21 stability. In addition, we also investigate the role of RelA's threonine 505 residue in the control of FMN2. Our results identify FMN2 as a crucial protein involved in the control of p21.

Introduction

The tumor suppressor pathway mediated by p14ARF is known to respond to oncogene activation, and it is crucial for the induction of one of the most important anticancer proteins in mammals, p53.¹ p53 activates fundamental cellular responses for the clearance of malignant cells, including apoptosis and cell cycle

arrest.² Cell cycle arrest under these conditions is dependent on p53 inducing the transcription of the cyclin-dependent kinase inhibitor p21. This pathway has been extensively studied both molecularly and genetically, and its physiological relevance has been clearly demonstrated in vivo.^{1,2} We have recently identified the human FMN2 protein as a novel player in this process using an unbiased proteomic screen for proteins changed in the nucleolus following induction of p14ARF (Fig. 1). Our results identified a mechanism by which p14ARF activation modulates NFκB activity, to increase transcription of the FMN2 gene. Furthermore, we have found that increased FMN2 expression is required to stabilize the cyclin-dependent kinase inhibitor p21 protein and thereby allows p21 expression to increase following p14ARF induction.³

Induction of p21-Increased Transcription and Enhanced Stability

It is known that p21 is induced transcriptionally by the tumor suppressor p53.^{2,4} However, gene expression can also be modulated at the protein level by altering the rate of either translation and/or protein degradation, as seen for p53 itself. In this case, for a protein to increase in abundance, the level of its translation must be higher than the level of its degradation. Higher translation rates can of course be achieved by increasing the abundance of transcripts, and previously induction of the cell cycle inhibitor p21 downstream of p14ARF was primarily ascribed to p53-dependent transcriptional

upregulation of the p21 gene. However, our analysis has now identified that a key part of the mechanism regulating p21 protein expression is not just transcription, but also control of p21 protein stability. We have identified in human cells that induction of p14ARF results in increased levels of both FMN2 mRNA and protein. We showed that the presence of the human FMN2 protein alone is sufficient to increase p21 protein levels without altering p21 mRNA. Our data suggest that FMN2 prevents the action of both ubiquitin-dependent and -independent degradation pathways from acting on p21 (Fig. 1).

The coupling of both increased transcription and enhanced protein stability provides an efficient mechanism for the control of protein expression. This coupled mechanism allows for a rapid, large increase in the level of protein in response to a stimulus and, importantly, also allows for the protein level to fall rapidly again when the stimulus is removed. Our

recent proteomic studies have screened for human proteins that are targeted for rapid degradation and showed that proteins can have differential rates of turnover in separate subcellular compartments.⁵⁻⁷ We had also previously observed that p14ARF is one of the proteins in the nucleolus with a very fast turnover rate.⁸ This prompted us to search for target proteins in the nucleolus affected by p14ARF induction and led to our identification of FMN2. Our studies on the role of FMN2 illustrate how the control of protein degradation rates can play a key role in the molecular mechanisms regulating gene expression during fundamental cellular responses.

p21 Function in Cells

The p21 protein is known to contribute to multiple important mechanisms involved in the control of cell proliferation and the cell cycle. Perhaps the best known role for p21 is the inhibition of specific Cyclin-CDK complexes in the G₁ and G₂

phases of the cell cycle.^{9,10} However, p21 can also block the action of transcription factors such as E2F1 and c-Myc^{9,10} and inhibits the activity of ROCK kinases that are responsible for changes in the cytoskeleton.^{9,10} In addition, p21 has been reported to exert both positive and negative effects over the process of apoptosis.^{9,10} Mice that are genetically depleted of p21 are viable and show late onset of spontaneous tumors, indicating that p21 is not a typical tumor suppressor gene.^{9,10} Furthermore, depending on the genetic interaction tested and tissue type, depletion of p21 can result in either tumor suppression or in tumor-promoting effects.⁹ A molecular explanation for this confusing observation of dual and apparently opposing functions appears to reside in the cellular localization of p21. While the majority of the anti-tumorigenic functions of p21 are located in the nucleus, the pro-cancer functions are based in the cytoplasm.¹⁰ This suggests that there may be distinct functional pools

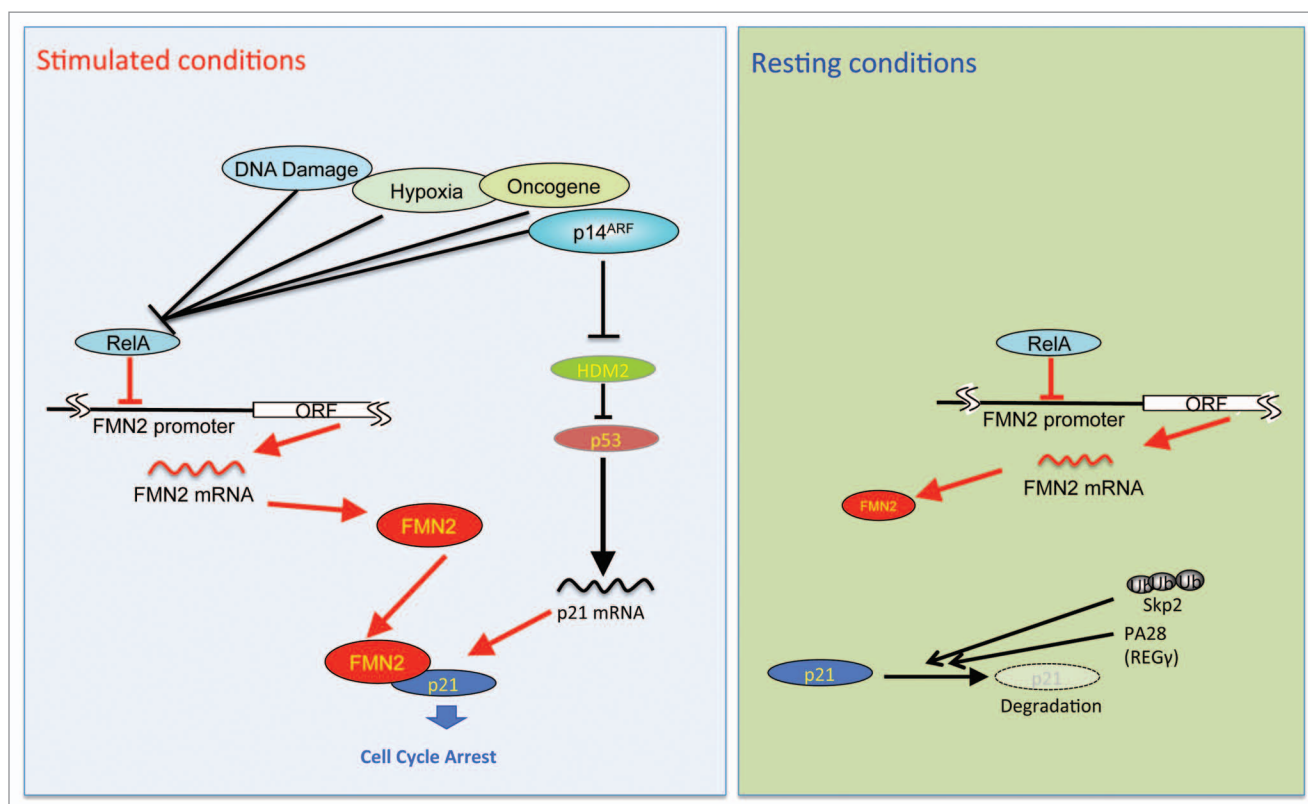


Figure 1. Schematic diagram depicting the role of FMN2 in the control of p21 expression. Under resting conditions, p21 mRNA is translated into protein but it is rapidly degraded by both ubiquitin-dependent and -independent pathways. Following either activation of p14ARF, DNA damage, or hypoxia responses, p21 transcription is increased, and p21 mRNA is translated into protein with concurrent increases in FMN2 transcription and protein expression. FMN2 prevents p21 degradation. High p21 levels then induce cell cycle arrest and inhibit proliferation.

of the p21 protein that can, at least in part, act on different targets and participate in different pathways in different subcellular compartments.

Multiple mechanisms affecting the subcellular localization of p21 have been found, including phosphorylation by AKT and MAPK.¹⁰ We have not yet investigated in detail the phosphorylation of p21 following FMN2 induction. However, using fluorescence microscopy we observed that most of the p21 signal is located in the nucleus of U2OS cells.³ Importantly, when U2OS cells were depleted of FMN2 by siRNA treatment following p14ARF induction, we observed an increase in the number of apoptotic cells and apoptosis markers. These effects could be mimicked using siRNA to deplete

p21 itself under the same conditions. These results support our hypothesis that FMN2 is required for p21-mediated cell cycle arrest and apoptosis inhibition. It will be interesting in the future to analyze also the effects of FMN2 depletion when different stimuli are tested, such as growth factor withdrawal and motility inducers.

What is the Functional Form of FMN2?

The function of the FMN2 protein has not been studied in detail previously, particularly not in human cells. Much of what has been reported on FMN2 comes from studies in mouse models, where it has been shown to be required for cell division, particularly in meiosis.¹¹ FMN2

is so named because it shares homology with the formin 1 (FMN1) gene, specifically the conserved formin repeat domain. In FMN2, this formin repeat region is present from the middle to the C terminus of the protein (Fig. 2A). Formin domains are required for nucleation of actin filaments in the cytoskeleton.¹² Surprisingly, therefore, our unbiased proteomic analysis and follow on studies revealed that p14ARF induction clearly resulted in increased levels of FMN2 mRNA and protein.

The data, however, do not distinguish whether it is the full-length FMN2 protein, including the formin repeat region, that accumulates in the nucleus and nucleolus following p14ARF induction. It is also possible that it is

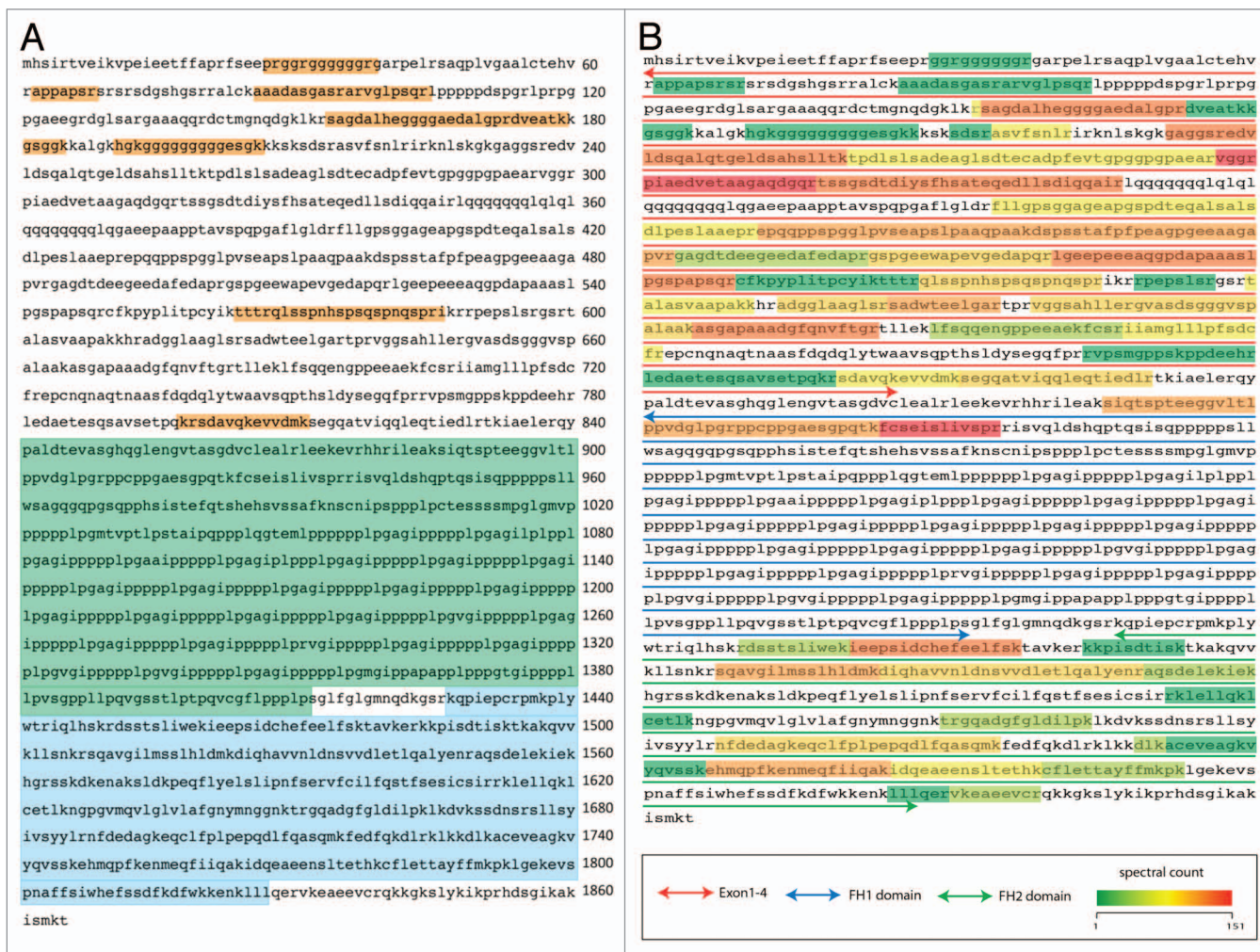


Figure 2. Proteomic coverage of FMN2. (A) FMN2 protein sequence with peptides identified through mass spectrometric analysis of nucleolar fraction highlighted in orange boxes. FH1 and FH2 domains were described by green and blue boxes, respectively. (B) FMN2 protein sequence with peptides identified by mass spectrometric analysis highlighted in different colors to reflect their spectral counts, i.e., the number of times they were sequenced. The protein was identified with a total sequence coverage of 46.5% and an N-terminal sequence coverage of 70.1%.

either a proteolytic cleavage fragment of full-length FMN2, or a separate isoform from the FMN2 gene, that is upregulated in the nucleus by p14ARF. As shown here, our mass spectrometry identification of FMN2^{13,14} has detected ~47% amino acid coverage across the whole protein sequence (Fig. 2B). Interestingly, however, mass spectrometric analysis of protein isolated from purified nucleoli identified mainly peptides from the N terminus of FMN2 (Fig. 2A, orange boxes). These data might suggest that either a specific isoform or processed fragment of FMN2 is induced by p14ARF, which could be generated either by protein cleavage, alternative splicing or through the use of an alternative transcription start site on the FMN2 gene.

To investigate this further, we have constructed a FMN2 fusion protein,

(GFP-FMN2-mCherry), that has different colored fluorescent tags at either end (Fig. 3A). When expressed in U2OS cells, this shows identical fluorescence localization of the green and red signals, with most of the signal in the cytoplasm. However, upon p14ARF induction, we observe a clear change and separation of the respective green and red signals. Specifically, the GFP signal is detected in the nucleus and nucleolus, while the mCherry remains mostly located in the cytoplasm with minimal accumulation in the nuclear compartment (Fig. 3B). The complementary result was observed when the positions of the GFP and mCherry tags were swapped between the N and C termini of FMN2 (data not shown).

These data thus support the hypothesis that specifically the N-terminal protein sequence of FMN2 is accumulated in the

nucleus following p14ARF induction. As this effect is observed using an exogenous fusion construct containing FMN2 cDNA, it is unlikely that the mechanism responsible requires either alternative splicing of FMN2 pre-mRNA, or a switch in the transcriptional start site at the FMN2 gene locus. This is consistent with the fact that we have not so far detected multiple forms of FMN2 mRNA whose ratio changes after p14ARF induction. We therefore currently favor the hypothesis that p14ARF may trigger processing of full-length FMN2 to generate one or more N-terminal fragments that translocate to the nucleus. A cleavage model that separates the unique N-terminal sequences from the actin-binding formin repeats can account for the ability of FMN2 to translocate into the nucleus. It will be interesting therefore to test this hypothesis in the future and to

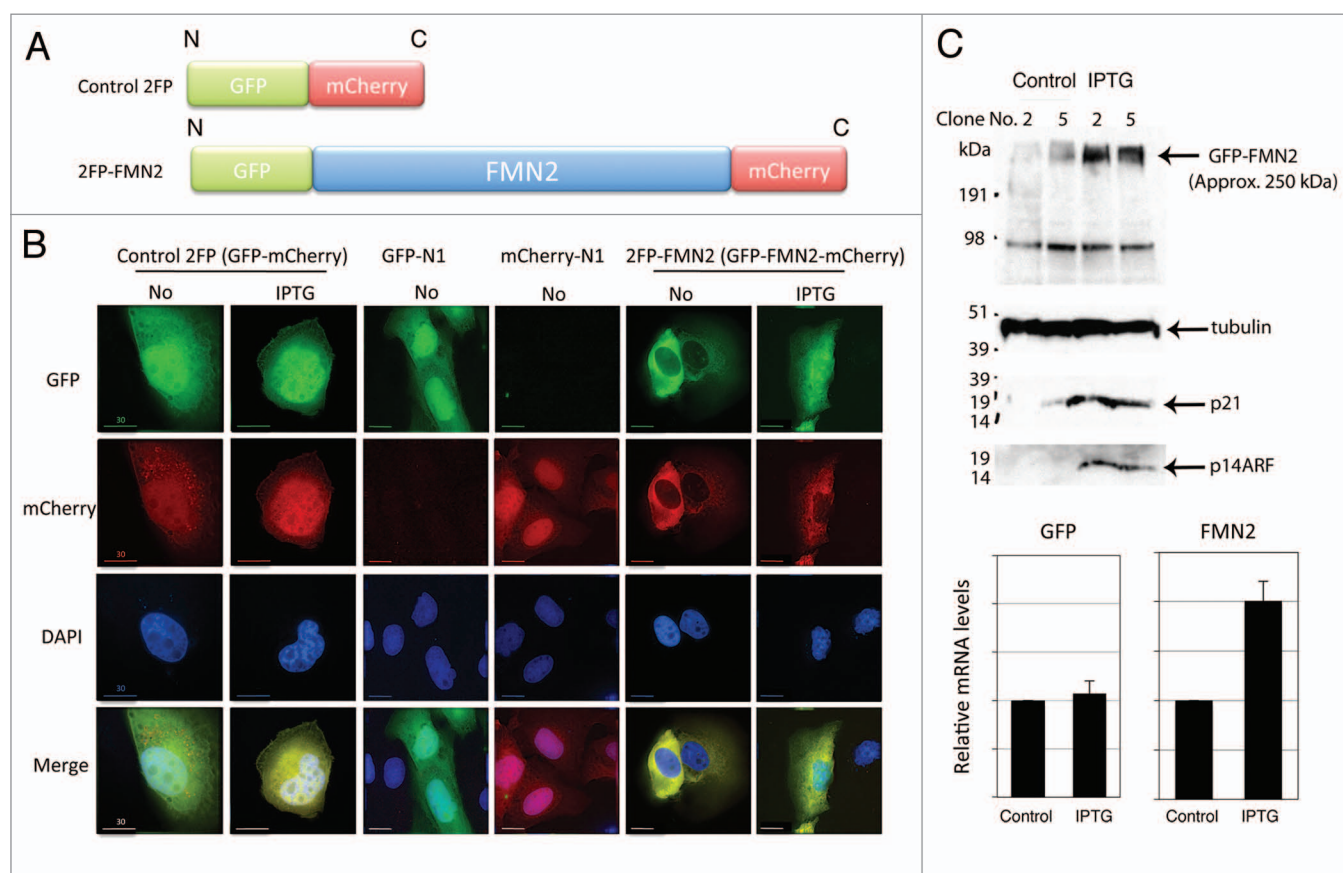


Figure 3. The N terminus of FMN2 is retained in the nucleus following p14ARF induction. (A) Schematic diagram of the 2FP-FMN2 expression plasmid, 2-Fluorescent protein tagged expression construct. (B) NARF2 cells were transfected with GFP-mCherry (Control 2FP) or GFP-FMN2-mCherry (2FP-FMN2) expression constructs prior to IPTG treatment for 24 h. Either GFP or mCherry expression plasmid was transfected as a control (GFP control and mCherry control). Cells were fixed and analyzed by microscopy for the localization of the different fluorescent proteins. Nuclei are stained with Hoescht. Scale bar indicates 30 μ m. (C) Two GFP-FMN2 stable cell lines (clones 2 and 5), which constitutively express GFP-tagged FMN2 protein, were established using NARF2 as the parental cell line. Western blotting (top panel) and quantitative RT-PCR (bottom panel) were performed to characterize GFP-FMN2 expression. p14ARF was induced for 24 h.

determine whether it is indeed a distinct N-terminal form of FMN2, rather than the full-length protein, that mediates the stabilization of p21.

Potential Role for the ATR/ATM Phosphorylation Events in FMN2

Specific phosphorylation sites on the FMN2 protein were identified within a large group of target sites on many proteins seen in a previous large-scale study of the DNA-damage checkpoint kinases ATR and ATM in human cells.¹⁵ Our analyses have further demonstrated that FMN2 expression is responsive to DNA damage.³ Given our previous results indicating a connection between p14ARF and ATR,¹⁶ it would be interesting to investigate if phosphorylation of FMN2 changes following ARF activation and to test the potential functional consequences for such modifications. As yet, we do not know whether the ATR/ATM phosphorylation sites detected on FMN2 are of functional significance. Interestingly, however, these phosphorylation sites also lie in the N terminus of the FMN2 protein, within the region we find in the cell nucleus following p14ARF induction.

One possible effect of this phosphorylation could be to increase the stability of the FMN2 protein itself. Our data have demonstrated that FMN2 protein levels increase upon inhibition of the proteasome.³ In support of this hypothesis, analysis of GFP-FMN2 stable cell lines, which constitutively express GFP-FMN2 protein driven by the CMV promoter, demonstrated that GFP-FMN2 proteins are stabilized by ARF induction at the protein level, but not at the transcriptional level (Fig. 3C). So far, there are no published data available on the half-life of FMN2 or any information on mechanisms that may regulate its stability. We have found that FMN2 levels respond to a wide variety of checkpoint activating stimuli, including activation of p14ARF and different oncogenes, as well as treatment of cells with etoposide, UV radiation, and hypoxia. This would suggest that FMN2 responds to both ATR- and ATM-mediated checkpoint functions in the cell. Further analysis of the functional significance of increased

FMN2 levels under these different stress conditions would be very interesting and may reveal additional roles for FMN2 in different cellular processes.

Potential Targets for FMN2

Our study identified p21 as a protein that is protected from degradation when FMN2 levels increase. Even though the levels of other labile proteins, including p53, Hdm2, and HIF-1, were not altered when FMN2 levels increased, it is possible that FMN2 can bind to and/or protect additional proteins in the human proteome. Therefore, to identify potential additional pathways that FMN2 may be involved in, it will be important to carry out further protein-protein interaction studies. Differential expression screens, either in the presence or absence of FMN2, at both the mRNA and proteomic level, would also be helpful and could give new insights into the functions and biological roles of FMN2 in human cells. We have shown that increased FMN2 levels protect p21 from both ubiquitin-dependent and -independent degradation pathways.³ However, it is possible that any additional proteins protected by FMN2 might be degraded primarily by one of these pathways. Again, additional research is required to answer this question.

Evolutionary Conservation of FMN2 and p14ARF Pathways

So far all of our analyses on p14ARF and FMN2 have been obtained using human cells. However, most of the previous published data on the FMN2 protein relates to studies on mouse cells. In the case of p14ARF, it is well established that human and mouse ARF proteins differ both in sequence and function.¹ Furthermore, some studies have shown that the INK4a/ARF locus suffers asymmetric evolution, with the ARF sequence in particular changing very rapidly from species to species.¹⁷ For example, while the mouse protein is 19 kDa, the human ortholog is only 14 kDa, while chicken ARF is even smaller,¹⁸ suggesting considerable diversity between species.

As mentioned before, most of the functions reported previously for the

FMN2 protein are derived from studies on mouse cells. While both the human and mouse FMN2 genes contain the Formin homology domain, some divergence is apparent between the human and mouse FMN2 sequences and even more with other species, such as chicken. While our data have revealed a novel function for FMN2, in particular for the N terminus of the protein, in the regulation of p21, we have not determined if this function of FMN2 is conserved in either mouse or other species. Given that the ARF proteins are rapidly evolving and show some different functions in different species, it is difficult to predict whether the role of FMN2 in the control of p21 expression levels is likely to be an evolutionary conserved feature. However, given that p21 itself has links to the control of the cytoskeleton, it is possible that FMN2 deletion in mice might impact on p21 levels and/or affect one or more pathways involving p21. Future detailed analyses in mice and other species are therefore required to answer this question.

Clinical Relevance of FMN2 as a Marker of Oncogene Activation, DNA Damage, or Hypoxia

Given that FMN2 expression is increased by multiple stimuli, including activation of p14ARF, DNA damage, and hypoxia, it is tempting to speculate that the FMN2 protein could provide a novel biomarker for diseases where these pathways are active. We have observed by analyzing the public databases in Oncomine (<http://www.oncomine.org>) that FMN2 mRNA is elevated in certain types of human cancer, such as melanoma and brain cancer, but reduced in other types of cancer, such as prostate. Furthermore, FMN2 was recently found to be overexpressed in 95% of pre-B acute lymphoblastic leukemias.¹⁹ These apparently opposing findings on FMN2 levels in different types of cancer are similar to what has also been observed for p21. Thus, while p21 levels are reduced in a number of cancer types, including colorectal cancer, it is also found to be overexpressed in a different subset of malignancies, such as gliomas.⁹ Further investigation of the levels of p21 and FMN2 at the protein level will

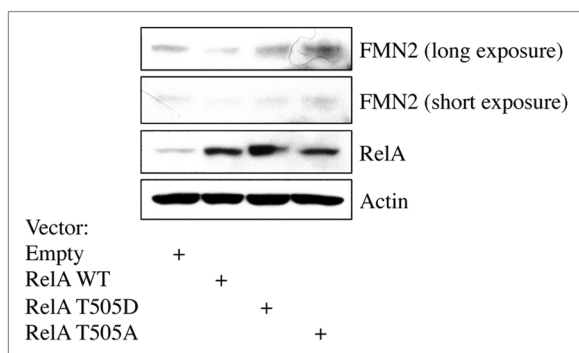


Figure 4. Role of the threonine 505 residue of RelA in the control of FMN2 expression. U2OS cells were transfected either with empty vector, or with RelA expression constructs, including wild-type, threonine 505 mutated to alanine (T505A), or threonine 505 mutated to aspartate (T505D), for 48 h prior to harvest. Whole-cell lysates were analyzed by western blot using the indicated antibodies.

therefore be required to establish whether the respective levels of these proteins are connected in any particular tumor type.

Role of Threonine 505 of RelA in the Regulation of FMN2 Gene Expression

We have previously identified a role for p14ARF in the control of NF κ B activity.^{16,20,21} In particular, we reported the phosphorylation of a key residue, threonine 505 (T505) on one of the NF κ B subunits, RelA, as being phosphorylated by Chk1 in an ATR-dependent manner.^{20,21} Recently, we demonstrated that expression of RelA carrying a mutation of T505 to alanine changes the growth, migration, and survival properties of the cell, further substantiating the relevance of this modification.²² Given that we have found that p14ARF-mediated induction of FMN2 is dependent of NF κ B, it would be interesting therefore to determine if in this case, phosphorylation of RelA on T505 was also involved in the regulation of this novel NF κ B target. To test this, we transiently overexpressed in U2OS cells, either wild-type RelA or mutant RelA forms, at the T505 residue, i.e., a non-phosphorylatable T505A and a phospho-mimic T505D (Fig. 4). Overexpression of wild-type RelA resulted in a modest reduction in the level of FMN2 protein. However, mutation of threonine 505 in RelA to either alanine, or aspartic acid, prevented the repression of FMN2 (Fig. 4), suggesting that this site may be important for RelA-mediated repression of FMN2.

The mechanism by which FMN2 is transcriptionally induced involves the displacement of RelA from the FMN2 promoter following p14ARF induction.³ This is a different mechanism to the one we observed for other genes such as Bcl-xL.^{21,23} An additional difference is the transcriptional outcome of the NF κ B modulation. While p14ARF induced the repression of Bcl-xL, it induces the increased expression of FMN2. Post-translational modifications on RelA could change the affinity of this transcription factor toward different promoters, resulting in increased binding at promoters such as Bcl-xL and decreased binding at FMN2. Phosphorylation of T505 could be such a modification. However, it seems more than likely that additional modifications may occur that induce this behavior of RelA, because our previous studies demonstrated that UV and etoposide do not induce RelA phosphorylation on T505.^{21,23} Ideally, analysis of the post-translation modification of RelA specifically bound at a given promoter, rather than the total cellular pool of RelA, would be required to fully elucidate the mechanisms governing RelA function.

Overall, our data have identified the human FMN2 protein as a novel regulator of the cyclin-dependent kinase inhibitor p21. Transcription of the FMN2 gene is induced in response to a variety of stimuli that induce cell cycle arrest by a mechanism involving displacement of the NF κ B subunit RelA from binding sites upstream of the FMN2 locus. These data demonstrate that FMN2 is a new target

gene for regulation by NF κ B. Modulation of FMN2 expression has a direct impact on the protein levels of p21 and therefore can contribute to the cellular responses affecting cell cycle arrest and/or cell death. Given the wide variety of stimuli we have found that can induce an increase in FMN2 levels, it is tempting to speculate that FMN2 could be useful in the clinic as a marker for the activation of oncogenes, DNA damage, and hypoxia in cells and tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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