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Keratin 23 is a PPARA-dependent, MYC-amplified oncogene that promotes hepatocyte proliferation

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Abstract

Chronic activation of the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARA) promotes MYC-linked hepatocellular carcinoma in mice. Recent studies have shown that MYC can function as an amplifier of transcription where MYC does not act as an 'on-off' switch for gene expression, but rather accelerates transcription rates at active promoters by stimulating transcript elongation. Considering the possibility that MYC may amplify the expression of PPARA target genes to potentiate cell proliferation and liver cancer, gene expression was analyzed from livers of wild-type and hepatocyte-specific *Myc* knockout mice ($Myc^{\Delta Hep}$) treated with the PPARA agonist Wy-14643. A subset of PPARA target genes was amplified in the presence of MYC, including keratin 23 (*Krt23*). The induction of *Krt23* was significantly attenuated in $Myc^{\Delta Hep}$ mice and completely abolished in *Ppara*-null mice. Reporter gene assays and chromatin immunoprecipitation confirmed direct binding of both PPARA and MYC to sites within the *Krt23* promoter. Forced expression of KRT23 in primary hepatocytes induced cell cycle-related genes. These data indicate that PPARA activation elevates MYC expression which in turn potentiates the expression of select PPARA target genes involved in cell proliferation. Finally, KRT23 protein are highly elevated in human hepatocellular carcinomas. These results revealed that MYC-mediated transcriptional potentiation of select PPARA target genes, such as *Krt23*, may remove rate-limiting constraints on hepatocyte growth and proliferation leading to liver cancer.

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AUTHOR CONTRIBUTIONS

D.K., C.N.B., S.T., T.Y., G.X., A.Q., and T.K., performed the research and analyzed the data. D.K., C.N.B., T.K., A.Q., and F.J.G. designed and supervised the research. D.K., C.N.B., and F.J.G. wrote the manuscript.

This paper is dedicated to the memory of Taehyeong Kim, Ph.D.

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Keywords

KRT; K23; Cytokeratin; cell cycle; c-MYC; HCC

Introduction

Peroxisome proliferator-activated receptor alpha (PPARA) is a ligand activated transcription factor and key mediator of lipid metabolism. PPARA agonists are considered 'classic' non-genotoxic carcinogens, and chronic PPARA activation causes hepatocyte proliferation, hepatomegaly, and eventually hepatocellular carcinoma (HCC) in rodents.[1, 2] Studies have shown the *Myc* oncogene contributes to agonist-induced hepatocyte proliferation. MYC upregulation occurs indirectly *via* the PPARA-mediated downregulation of *miRlet7c-1*, which targets *Myc* mRNA for degradation.[3] The contribution of MYC to hepatocyte proliferation was confirmed *in vivo* using liver-specific *Myc* knockout mice ($Myc^{\Delta Hep}$) treated with the potent PPARA agonist Wy-14643. Interestingly, agonist treated $Myc^{\Delta Hep}$ mice were resistant to, but not completely protected from, hepatocyte proliferation and hepatomegaly.[4] Additionally, $Myc^{\Delta Hep}$ mice were only partially protected from *N*-nitrosodiethylamine (DEN)-induced HCC.[4] DEN-treated *Myc* knockout mice had less than half the incidence of preneoplastic foci and adenomas. These data suggest that, in these models, MYC does not initiate cell proliferation but rather potentiates the proliferative effects initiated by alternate mechanisms.

A model of MYC action was recently proposed where MYC functions as a general amplifier of transcription, further elevating expression of transcriptionally active genes.[5, 6] In this model, MYC binding accelerates transcription rates by facilitating the release of RNA polymerase from its 'paused' state, thereby promoting transcript elongation.[7, 8] This model originated from the fact that MYC targets vary greatly between cell types and the lack of target gene overlap makes it difficult to ascribe a distinct gene signature associated with MYC activation.[9–11] Studies supporting MYC amplification of gene expression have primarily relied on microarrays and next generation sequencing data derived from cell lines where MYC levels or activity is artificially modulated.[6] Other studies have suggested that MYC does not universally amplify transcriptionally-active genes, and instead activates discrete sets of genes in a tumor-, tissue-, and cell type-specific fashion.[10–12] A major obstacle for testing MYC amplifier activity *in vivo* is the need for coordinated control of both a defined set of target genes and MYC expression levels. Pharmacological activation of the transcription factor PPARA in combination with hepatocyte-specific *Myc* knockout mice affords a unique opportunity to test the MYC amplifier model *in vivo*. *Myc* ablation only provided partial protection against the proliferative effects of prolonged PPARA activation.[1, 2, 4] This observation could be explained by an underlying mechanism involving the MYC-mediated amplification of PPARA target genes. Transcriptional amplification of select genes by MYC may therefore play a major role in agonist-induced HCC models.[13]

The present studies in hepatocyte-specific *Myc*-null mice and *Ppara*-null mice treated with the PPARA agonist Wy-14643 identified several PPARA target genes that were significantly amplified by MYC. Notably, *Myc*-dependent gene amplification was not universal as many

'classic' PPARA target genes involved in the control of lipid metabolism were unaffected by *Myc* status. Notably, keratin 23 (*Krt23*) was upregulated over forty-fold in response to PPARA activation and the response was markedly attenuated in liver-specific *Myc*-null mice. KRT23 is type I acidic cytokeratin protein that is highly expressed in several human cancers suggesting that it may contribute to PPARA-mediated hepatocellular proliferation.[14–17] The current study examined the hypothesis that MYC directly acts as a transcriptional amplifier of select PPARA target genes, including *Krt23*, and that upregulation of *Krt23* promotes hepatocyte proliferation and potentially HCC.

Materials and Methods

ANIMALS

Myc$^{fl/fl}$ mice were provided by John M. Sedivy (Brown University) and described previously.[18] For temporal hepatocyte-specific disruption, *Myc*$^{fl/fl}$ mice were crossed with mice harboring the Cre-ER^{T2} recombinase driven by the albumin promoter, designated *Alb-Cre-ER*^{T2}.[19] obtained from Pierre Chambon and Daniel Metzger (University of Strasbourg), to yield the *Myc*$^{fl/fl,ERT2\text{-}Cre}$ mouse line. The resulting hepatocyte-specific *Myc* knockout mice are subsequently referred to as *Myc*$^{\Delta Hep}$. For activation of the *Alb-Cre-ER*^{T2} driven CRE recombinase, male mice were injected intraperitoneally with 2 mg tamoxifen per mouse while being fed a tamoxifen diet (1 mg/kg, Dyets Inc., Bethlehem, PA) for 3 days. For Wy-14643 treatment, mice were fed for three days with tamoxifen diet then switched to a grain-based diet containing 0.1% Wy-14643 (Bio-Serv, Frenchtown, NJ), while receiving 2 mg tamoxifen by intraperitoneal (IP) injection every other day for an additional 3 days. *Ppara* wild-type (*Ppara*$^{+/+}$) and conventional *Ppara*-null mice (*Ppara*$^{-/-}$) used in this study were described previously.[20] For Wy-14643 studies, mice were provided a grain-based control diet or matched diet containing 0.1% Wy-14643 for two days. For the MK-8745 study, wild-type mice were given 15 mg/kg/day MK-8745 by intraperitoneal injection (IP) and provided either a 0.1% Wy-14643 diet or matching control chow diet for 3 days. Male 6- to 8-weeks-old mice were used for all studies and all mouse strains were on the C57BL6N background. Mice were housed in light and temperature-controlled rooms and provided water and pelleted chow *ad libitum*. All animal experiments were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care international guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

SUPPLEMENTAL METHODS

Cell culture; Histological and immunohistochemical (IHC) analysis; Production of recombinant KRT23-expressing adenovirus; Preparation of adeno-associated virus (AAV) short hairpin (sh) RNA of KRT23; Primary hepatocyte isolation and adenoviral transduction; Western blot analysis; Quantitative reverse transcription PCR (qRT-PCR) assays; Luciferase reporter assays; Chromatin immunoprecipitation (ChIP) assays; Computational and Statistical analyses

Results

HEPATIC KERATIN 23 (KRT23) AND OTHER PPARA TARGET GENES ARE AMPLIFIED BY MYC

Liver-specific *Myc* knockout mice are resistant to, but not completely protected from, PPARA-induced hepatocyte proliferation and DEN-induced HCC.[4] Considering the possibility that MYC may act as an amplifier of certain PPARA target genes, microarray data from a previous study was re-analyzed (GSE43842).[4] The analysis of the array data from livers of floxed wild-type ($Myc^{fl/fl}$) and $Myc^{\Delta Hep}$ mice treated with Wy-14643 revealed that several PPARA target gene mRNAs were significantly amplified in the presence of MYC (Fig 1A; upper panel) while many others were not impacted by MYC status (Fig. 1A; lower panel). $Myc^{fl/fl}$ and $Myc^{\Delta Hep}$ mice were treated with Wy-14643 for 48 h to confirm *Myc*-dependent changes in *Krt23* mRNA and protein expression. As expected, *Myc* mRNA was upregulated in $Myc^{fl/fl}$ but ablated in $Myc^{\Delta Hep}$ mice treated with Wy-14643 (Fig. 1B). *Krt23* mRNA expression was increased in both wild-type and $Myc^{\Delta Hep}$ mice in response to PPARA activation, while levels were significantly blunted in $Myc^{\Delta Hep}$ mice (Fig. 1B). Western blotting revealed that KRT23 protein levels were also lower in agonist-treated $Myc^{\Delta Hep}$ mice (Fig. 1C) and densitometry confirmed a statistically significant difference in protein expression (Fig. 1D). To test whether Krt23 expression can promotes hepatocyte proliferation independent of MYC, an adeno-associated virus (AAV) expressing Krt23 vector (AAV-Krt23) was generated. $Myc^{F/F}$ and $Myc^{\Delta Hep}$ mice were placed on a Wy-14643 diet for five days and then injected with AAV-Krt-23. Wy-14643 treatment and AAV-Krt23 induced more pronounced hepatomegaly and cell proliferation when compared to Wy-14643 or AAV-Krt23 alone (Supporting Fig. S1A and 1-H). However, hepatocyte-specific loss of MYC expression did not significantly attenuate hepatomegaly or *Krt23* mRNA expression (Supporting Fig. S1B). KRT23 and MYC protein levels were significantly increased by the combination of Wy-14643 and AAV-Krt23 compared to Wy-14643 treatment or AAV-Krt23 overexpression alone. Surprisingly, KRT23 protein produced from the AAV-Krt23 was significantly attenuated by liver-specific *Myc* knockout through a yet to be determined mechanism (Supporting Fig. 1C-1E). These data support the view that hepatic *Krt23* expression is PPARA-dependent and amplified by, but not dependent on MYC.

MYC AMPLIFICATION OF PPARA TARGET GENES IS NOT UNIVERSAL

To assess MYC-mediated amplification of select PPARA target genes, wild-type, $Myc^{\Delta Hep}$ and *Ppara*-null mice were treated with Wy-14643 then livers harvested and mRNAs quantified. Fatty acid 2-hydroxylase (*Fa2h*), otopetrin 1 (*Otop1*), striatin interacting protein 2 (*Strip2*; a.k.a. *Fam40b*), C-X-C motif chemokine receptor 1 (*Cxcr1*), and *Krt23* mRNAs were induced by Wy-14643 in wild-type mice, attenuated in $Myc^{\Delta Hep}$ mice, and completely blocked in *Ppara*-null mice, confirming the expression patterns observed in the microarray data (Fig. 1E). In contrast, expression of the 'classic' PPARA target gene mRNAs acyl-coenzyme A oxidase 1 (*Acox1*), cytochrome P450 family 4 subfamily A polypeptide 10 (*Cyp4a10*), and enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase (*Ehhadh*) was completely PPARA-dependent and unaffected by *Myc* status (Fig. 1F). These data in Wy-14643 treated *Ppara*-null and $Myc^{\Delta Hep}$ mice substantiate that select PPARA target genes are further amplified by MYC.

KRT23 RESPONSE IS PPARA-DEPENDENT AND LIVER-SPECIFIC

Since PPARA agonist-induced cell proliferation is hepatocyte-specific and not observed in other tissues, genes that contribute to hepatocyte proliferation would be expected to increase only within the liver. *Myc*, *Krt23,* and *Cyp4a14* mRNA levels were examined in tissues from wild-type and *Ppara*-null mice treated with the PPARA agonist Wy-14643. *Cyp4a14,* a well-characterized PPARA target gene, was used as a positive control to assess PPARA activation. PPARA was activated to varying degrees in all tissues as evaluated by *Cyp4a14* mRNA expression (Fig. 2A). Induction of *Myc* and *Krt23* mRNAs by Wy-14643 was liver-specific and not observed in *Ppara*-null mice indicating that their expression is PPARA-dependent (Fig 2B and C). Basal *Krt23* levels were also significantly decreased in *Ppara*-null mice compared to wild-type mice (Fig. 2C). Induction of hepatic KRT23 protein expression by Wy-14643 administration was further confirmed by Western blotting (Fig. 2D). Histological examination of liver sections by H&E staining and immunohistochemistry (IHC) revealed pronounced hepatocyte swelling in Wy-14643 treated animals. KRT23 staining was primarily cytosolic and was increased significantly in response to Wy-14643 treatment when compared to controls; staining was markedly decreased in $Myc^{\Delta Hep}$ mice (Fig. 2E and F). Knockdown of *Krt23* with AAV-shRNA-*Krt23* inhibited Wy-14643 induced hepatocyte proliferation and hepatomegaly (Supporting Fig. S2). Combined treatment of mice with Wy-14643 and AAV-KRT23 significantly increased the number of BrdU-positive hepatocytes (Supporting Fig. S1G and 1H). These data show that *Myc* and *Krt23* expression in response to PPARA activation was liver-specific, supporting a role for these proteins in hepatocyte proliferation.

KRT23 IS THE PRINCIPAL KERATIN UPREGULATED DURING PPARA-INDUCED HEPATOCYTE PROLIFERATION

Historically keratins are considered structural filament forming proteins but more recently studies have shown these proteins facilitate cell signaling pathways by acting as scaffolds.[21] A dendrogram of KRT protein sequences depicts sequence homology of keratins expressed in liver (Fig. 3A). *Krt7*, *Krt8*, *Krt10*, *Krt18*, *Krt19*, and *Krt20* are commonly used as prognostic and diagnostic markers for liver disease and cancer.[21, 22] Notably, *Krt10* and *Krt19* are used as diagnostic markers for liver tumor pathology.[23] As expected, *Krt23* mRNA expression increased sixty-fold in wild-type mice and levels were unchanged in *Ppara*-null mice treated with Wy-14643. *Krt4* and *Krt28* mRNA levels also increased but to much lower extent, three-fold and two-fold, respectively, in response to PPARA activation. (Fig. 3B). Ductular reactions are a characteristic response to liver injury and correlate with liver disease severity. KRT7, KRT8, KRT18, KRT19, and KRT23 are used as markers for ductular reaction. To determine whether Krt23 affects ductular reactions, *Krt7* and *Krt19* mRNA levels were measured in primary hepatocytes infected with adenovirus expressing KRT23. Results revealed that the overexpression of KRT23 does not impact the expression of *Krt7, Krt8, Krt18*, or *Krt19* mRNA (Fig. 3C) suggesting that KRT23 has a minimal role in the development of ductular reactions.

PPARA DIRECTLY REGULATES KRT23 BY BINDING TO PROMOTER RESPONSE ELEMENTS

Genomatix MatInspector (Genomatix, Munchen, Germany) was used to bioinformatically identify potential peroxisome proliferator response elements (PPREs) within the *Krt23* promoter. Five PPRE sites were identified within 1 kb upstream of the *Krt23* transcriptional start site (TSS) (Fig. 4A). Predicted MYC binding sequences, known as an enhancer box (Ebox), are shown for reference. Inserts (A-C) containing the predicted PPRE sites were synthesized and cloned into the pGL4.11 reporter and luciferase assays performed to confirm direct PPARA binding within the *Krt23* promoter. Another PPRE-luc construct containing a *Acox1* PPRE repeat was used as a positive control and empty pGL4.11 plasmid was used as a negative control. Luciferase signal was significantly elevated in primary hepatocytes transfected with the pGL4.11-A construct which contained a single PPRE site, referred to as PPRE(1), indicating direct regulation by PPARA (Fig. 4B). pGL4.11-B and -C reporter signal was similar to empty vector. PPARA binding was also assessed by ChIP assays on chromatin isolated from livers of wild-type mice on control diet or treated with Wy-14643-treated using a PPARA antibody. The results revealed an enrichment using primer sets ‘A’ and ‘B’ (Fig. 4C). Enrichment was stronger with primer set ‘A’ which targets PPRE(1) in agreement with the luciferase data. Further enrichment was also observed at PPRE(1) after treatment with Wy-14643. Samples from *Ppara*-null mice on control diet were used as a negative control. Together these data indicate that PPARA regulates *Krt23* transcription primarily through direct binding of a PPRE site found within 100bp of the *Krt23* TSS.

MYC DIRECTLY REGULATES KRT23 BY BINDING TO EBOX SITES WITHIN ITS PROMOTER

Genomatix MatInspector also identified potential MYC binding Ebox sites within the *Krt23* promoter. Luciferase assays were performed to confirm the presence of functional MYC-binding sites. Three Ebox sites (1–3) were identified within 1 kb upstream of *Krt23* exon 1 and MYC-binding element reporter constructs were generated for each (Fig 5A). The PPRE(1) site was also included in all constructs. Constructs were transiently co-transfected with either empty vector (pBABE-empty) or a construct that expresses constitutively-activated MYC (pBABE-MYC) into primary hepatocytes.[3] Ebox 1, 2, and 3 contain functional MYC response elements (Fig. 5B). ChIP assays were then performed and the results quantitated showing that MYC can bind to all Ebox sites when compared to the negative (NEG) control primer sets that targeted a region around −2 kb upstream of the *Krt23* TSS. However, only Ebox 1 showed enrichment after Wy-14643 treatment (Fig. 5C and D).

KRT23 INDUCED G2/M-RELATED GENE EXPRESSION IN MOUSE PRIMARY HEPATOCYTES

To test the impact of KRT23 on cell cycle control, adenovirus expressing KRT23 was used to infect primary mouse hepatocytes to determine whether KRT23 might act as a liver-specific, MYC-amplified oncogene. KRT23 expression upregulated mRNAs encoded by G2/M-related genes such as cell division cycle 25C (*Cdc25c*), cyclin B1 (*Ccnb1*), cyclin B2 (*Ccnb2*), and cyclin dependent kinase 1 (*Cdk1*) (Fig. 6A). Interestingly, these mRNAs are unchanged in Hepa-1 cells infected with KRT23-expressing adenovirus (Supporting Fig.

S3). The 'classic' proliferation markers including *Ki67* and *Pcna* mRNAs were also significantly increased after forced KRT23 expression (Fig. 6A). Forced KRT23 expression in primary hepatocytes led to the upregulation of aurora kinase A (*Aurka*) and B (*Aurkb*), as well as many AURK-associated co-factors including microtubule nucleation factor (*Tpx2*), mitotic checkpoint serine/threonine kinase (*Bub1*), transforming, acidic coiled-coil containing protein 3 (*Tacc3*), and centromere protein A (*Cenpa*). AURKA/B target gene mRNAs such as centromere protein A (*Cdca8*), baculoviral IAP repeat-containing 5 (*Birc5*), and inner centromere protein (*Incenp*) were also significantly increased in KRT23-expressing primary hepatocytes (Fig. 6B). KRT23, CDC25C, and CCNB1 proteins were significantly elevated after infection (Fig. 6C). Upregulation of AURKA protein expression was also confirmed in KRT23-expressing cells (Fig. 6D). KRT23 is known to interact with and sequester the scaffold protein 14–3-3 epsilon (14–3-3ε), also known as YWHAE, which regulates many cellular processes including signal transduction and the cell cycle.[14] To verify the interaction between KRT23 and 14–3-3ε, liver lysates from Wy-14643-treated mice were used in KRT23 immunoprecipitation (IP) assays. Pull-downs were probed by Western blotting using a 14–3-3ε antibody and results support a strong interaction between KRT23 and 14–3-3ε (Fig. 6E).

WY-14643-INDUCED KRT23 EXPRESSION IS PARTIALLY REPRESSED BY AURORA KINASE INHIBITION

Previous studies have shown AURKA upregulates MYC and promotes cancer progression in the liver.[24] A regulatory loop may exist between MYC, KRT23, and AURKA, where MYC amplifies KRT23 expression which then promotes AURKA subsequently reinforcing MYC dysregulation. To test whether inhibition of AURKA signaling influences MYC and KRT23 expression, MK8745, a specific AURKA inhibitor, was used in the Wy-14643-induced hepatocyte proliferation model. Liver/bodyweight ratios and the number of BrdU-positive hepatocytes were increased by Wy-14643, and but not significantly inhibited by MK-8745 treatment (Supporting Fig. S4). *Myc* and *Krt23* mRNA expression were partially but significantly inhibited by MK-8745 treatment (Fig. 7A). Western blotting and quantitative densitometry indicated that the protein levels were also significantly decreased in response to treatment (Fig. 7B and C). In agreement with MYC downregulation, PPARA-dependent MYC-amplified genes were suppressed by AURK inhibition (Fig. 7D). A previous study found that the initial increase in MYC expression involved the PPARA-independent downregulation of *miRlet7c-1* which targets *Myc* mRNA.[3] These data suggest that MYC expression may be reinforced through an alternate mechanism involving KRT23. MYC amplifies *Krt23* expression then KRT23 increases AURKA signaling possibly through an interaction with 14–3-3ε. Studies have shown AURK signaling bolsters MYC expression.[24] By inhibiting AURK while PPARA is activated, MYC levels decreased and MYC-amplified gene expression was attenuated. Together these data provide evidence to support the MYC-mediated amplification of PPARA target genes, in particular *Krt23*, might contribute to agonist-induced hepatocyte proliferation possibly through a mechanism involving AURKA (Fig. 7E).

KRT23 EXPRESSION CORRELATES WITH HUMAN HCC PROGRESSION

Previous studies revealed that KRT23 levels were significantly increased in patients with steatohepatitis when compared to patients with steatosis or healthy liver[25], and elevated in HCC patient sera when compared to control sera.[26] To investigate whether KRT23 may contribute to human liver cancer, protein levels were measured in human liver tumor biopsies by western blot (Fig. 8A). MYC, CCNB1, and AURKA expression was also assessed. Expression of KRT23, MYC, CCNB1, and AURKA was elevated in tumor samples when compared to normal liver tissue. Western blot data was further supported by immunohistochemical analysis which showed elevated KRT23, MYC, and AURKA expression in human HCC sections (Fig. 8D). These results confirm KRT23 expression is also significantly elevated in human HCC. Moreover, expression levels roughly correlated with tumor progression indicating KRT23 may act as a useful diagnostic biomarker for human tumor progression.

Discussion

Keratins are conventionally thought of as structural proteins, but more recently several studies have revealed that they also play active roles in cell signaling pathways.[22, 23] KRTs also commonly serve as diagnostic and prognostic markers for cancer progression and clinical outcomes associated with a wide range of cancers.[23] To date, there is little information related to the functional consequences of KRT dysregulation.[21, 23] The current study identified KRT23 as a potentially novel PPARA-dependent and MYC-amplified liver-specific oncogene. Prolonged PPARA activation causes hepatocyte proliferation and HCC in rodents. The PPARA agonist Wy-14643 significantly induced *Krt23* mRNA and KRT23 protein expression in livers of wild-type mice. *Krt23* expression was unchanged in *Ppara*-null mice treated with a PPARA agonist indicating that *Krt23* induction is PPARA-dependent. Although *Krt23* expression was also upregulated in $Myc^{\Delta Hep}$ mice, gene response was much lower than in wild-type animals supporting the role of MYC as a possible amplifier for *Krt23*. These results indicate that *Krt23* expression is influenced by, but not dependent on, MYC expression. Considering the high degree of sequence homology, KRT23 and possibly other KRT family members may promote proliferation by similar mechanisms.

The underlying PPARA-MYC regulatory interactions controlling *Krt23* expression was further assessed by analyzing the *Krt23* promoter region. PPARA and MYC binding sites were bioinformatically predicted in the 1 KB region upstream of the *Krt23* TSS. The *Krt23* promoter contains five predicted PPARA binding sites, one of which was found to functional bind PPARA by reporter gene and ChIP assays. Notably, three MYC-binding Ebox sites were identified within 1 KB of the *Krt23* TSS. Luciferase reporter assays indicated transactivation by MYC at all three sites; these sites were also shown by ChIP to directly bind MYC. The identification of multiple functional MYC binding sites within a PPARA target gene is supports the possibility that some of these MYC-amplified PPARA target genes might play a role in agonist-induced hepatocellular proliferation leading to HCC. Several studies recently provided convincing support for a novel model of MYC action that represents a paradigm shift in our understanding of this important oncogene.[5, 6] In this

model, MYC does not act as an 'on-off' switch for gene expression, but rather accelerates transcription rates at active promoters by stimulating transcript elongation.[8] While the 'MYC amplifier model' was defined by cell culture studies, it was not certain that this hypothesis is physiologically-relevant, or whether amplification of an existing gene expression program might drive tissue-specific tumorigenesis. The present data indicate that the Wy-14643-mediated upregulation of *Krt23* is both PPARA-dependent and amplified by MYC.

Treatment of mice with carcinogens such as diethylnitrosamine causes HCC through activation of G2/M-related gene expression.[27] Thus, cell cycle-related gene expression was measured in mouse primary hepatocytes. Adenovirus-expressing KRT23 induced G2/M-related gene expression including *Cdc25c*, *Ccnb1*, *Ccnb2,* and *Cdk1*. Since Hepa-1 is a mouse hepatoma cell line, perhaps forced exogenous KRT23 expression is unable to further enhance cell cycle progression and proliferation.[28] Previous studies revealed that KRT23 interacts with 14–3-3 epsilon (14–3-3ε) in human colon cancer cells and that this interaction influences many cellular processes including cell cycle and apoptosis.[14] KRT23 immunoprecipitation using Wy-14643 treated mouse liver lysates confirmed the direct association of KRT23 with 14–3-3 *in vivo*.

Aurora kinases (AURKA) are known to control G2/M-related genes and mediate the oncogenic effects in HCC at least partially through the upregulation of MYC.[24] *Aurk* and *Aurk*-related gene expression was assessed in mouse primary hepatocytes infected with KRT23-expressing adenovirus. KRT23 induced both *Aurka* and *Aurkb* mRNA expression in primary hepatocytes. AURK-associated co-factors and downstream target gene mRNAs including *Tpx2*, *Bub1*, *Tacc3*, *Cenpa*, *Cdca8*, *Birc5,* and *Incenp* were significantly increased by KRT23 expression. Upregulation of AURK by KRT23 would then be expected to form a feed forward loop where KRT23 upregulates AURKA/B expression thereby further promoting *Myc* expression and the subsequent amplification of *Krt23* expression. Conversely, AURKA/B inhibition would be expected to decrease KRT23 expression through downregulation of MYC. To verify whether the inhibition of AURK suppresses KRT23, mice were treated with Wy-14643 and the AURKA inhibitor MK-8745. Wy-14643-induced *Krt23* and *Myc* mRNA expression were partially inhibited by MK-8745 treatment. Upregulation of PPARA-dependent, MYC-amplified target genes were also significantly attenuated by AURKA inhibition. KRT23 expression was also highly elevated in human hepatocellular carcinomas suggesting dysregulation may have clinical significance. These data support the view that the PPARA-dependent MYC-mediated amplification of KRT23 may involve a regulatory loop that includes aurora kinase signaling. Interestingly, 14–3-3 proteins act as a phosphodocking scaffold and mediate many phosphoregulatory pathways including those associated with aurora kinases.[29, 30] Moreover, 14–3-3ε is an AURK target and phosphorylation regulates 14–3-3 function.[29, 30] The interaction between KRT23, 14–3-3, and AURKA/B represents another potential regulatory mechanism that warrants further exploration.

The present studies demonstrate that MYC can act as a gene amplifier *in vivo* using a controlled system where a well-defined set of genes can be turned on and off by PPARA in the presence or absence of MYC. Furthermore, demonstrating that MYC-mediated

amplification of KRT23 contributes to cell proliferation underscores the pleiotropic mechanisms driving MYC-induced carcinogenesis in different cell and tissue types. As MYC is commonly thought of as ‘undruggable’ in a clinical setting, results from these studies would support that targeting MYC-amplified, tissue-specific, and seemingly unrelated transcription factors may represent therapeutically efficacious targets for MYC-associated tumors. These studies also highlight KRT23 as a potentially novel drug target and diagnostic biomarker for human liver tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS:

AURKA	aurora kinase A
ChIP	chromatin immunoprecipitation
Ebox	enhancer box
HCC	hepatocellular carcinoma
IP	immunoprecipitation
KRT23	keratin 23
PPARA	peroxisome proliferator-activated receptor alpha
PPRE	peroxisome proliferator-activated receptor response element
qRT-PCR	quantitative real-time polymerase chain reaction
TSS	transcription start site

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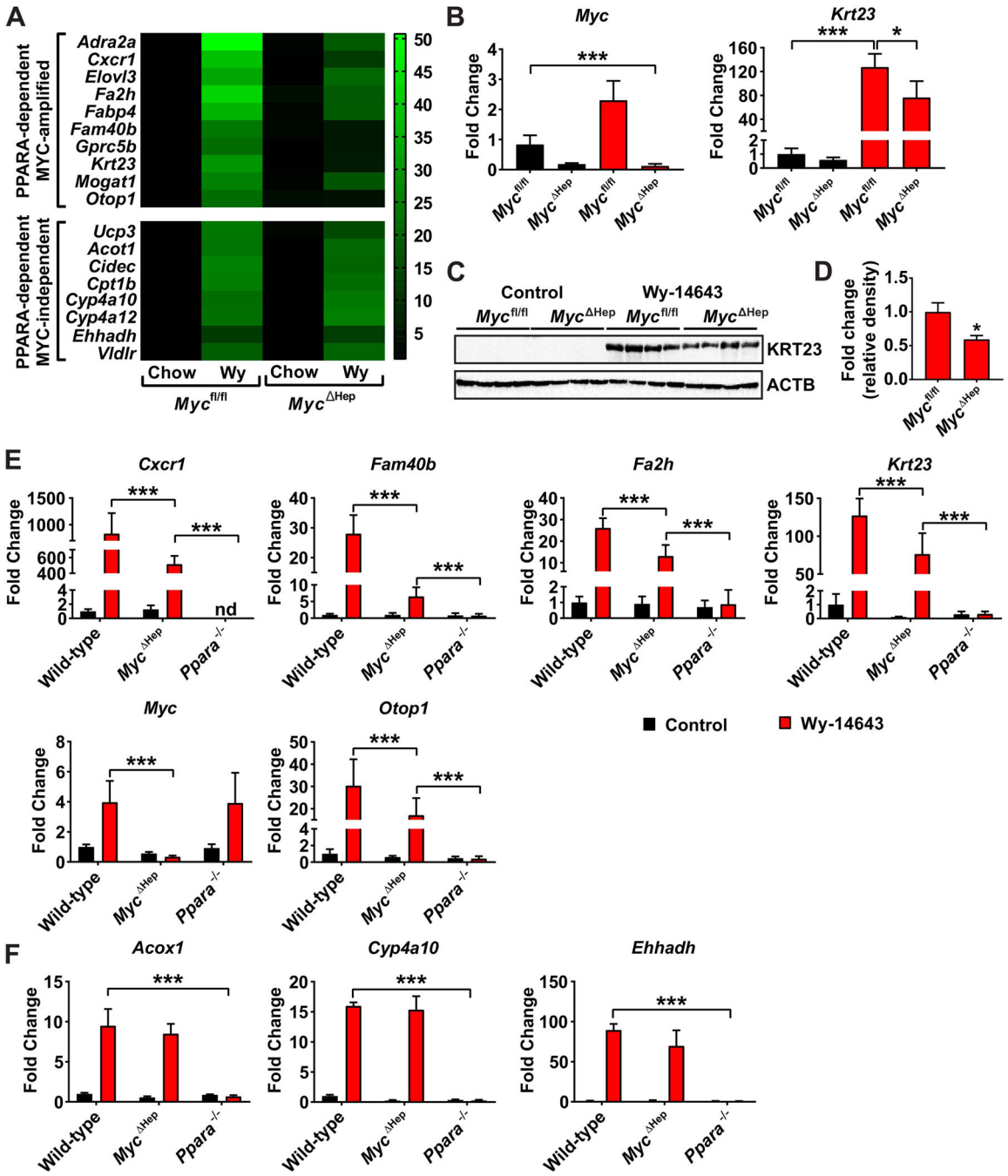

FIG. 1.

MYC expression amplifies select PPARA target genes. (A) Liver microarray data from *Myc*-floxed ($Myc^{fl/fl}$) and hepatocyte-specific *Myc*-null ($Myc^{\Delta Hep}$) mice treated with Wy-14643. Heatmap showing representative MYC-amplified genes from microarray data. Upper panel depicts PPARA target gene mRNAs amplified by MYC. Lower panel shows PPARA targets unaffected by MYC status. (B) *Myc* and *Krt23* expression in wild-type and $Myc^{\Delta Hep}$ mice treated with Wy-14643. (C) Western blotting analysis of KRT23 protein expression in liver from $Myc^{fl/fl}$ and $Myc^{\Delta Hep}$ mice treated with Wy-14643. (D) Densitometry analysis of

KRT23 protein levels. (E) Quantification of *Cxcr1*, *Fam40b*, *Fa2h*, *Krt23*, *Myc,* and *Otop1* mRNAs in wild-type, $Myc^{\Delta Hep}$, and *Ppara*-null mice treated with Wy-14643. (F) Quantification of PPARA-dependent MYC-independent *Acox1*, *Cyp4a10*, and *Ehhadh* mRNAs in wild-type, $Myc^{\Delta Hep}$, and *Ppara*-null mice treated with Wy-14643. Experiments were performed on at least five mice/group. Each data point represents the mean ± SD (ns, not significant; *, $p < 0.05$; ***, $p < 0.001$).

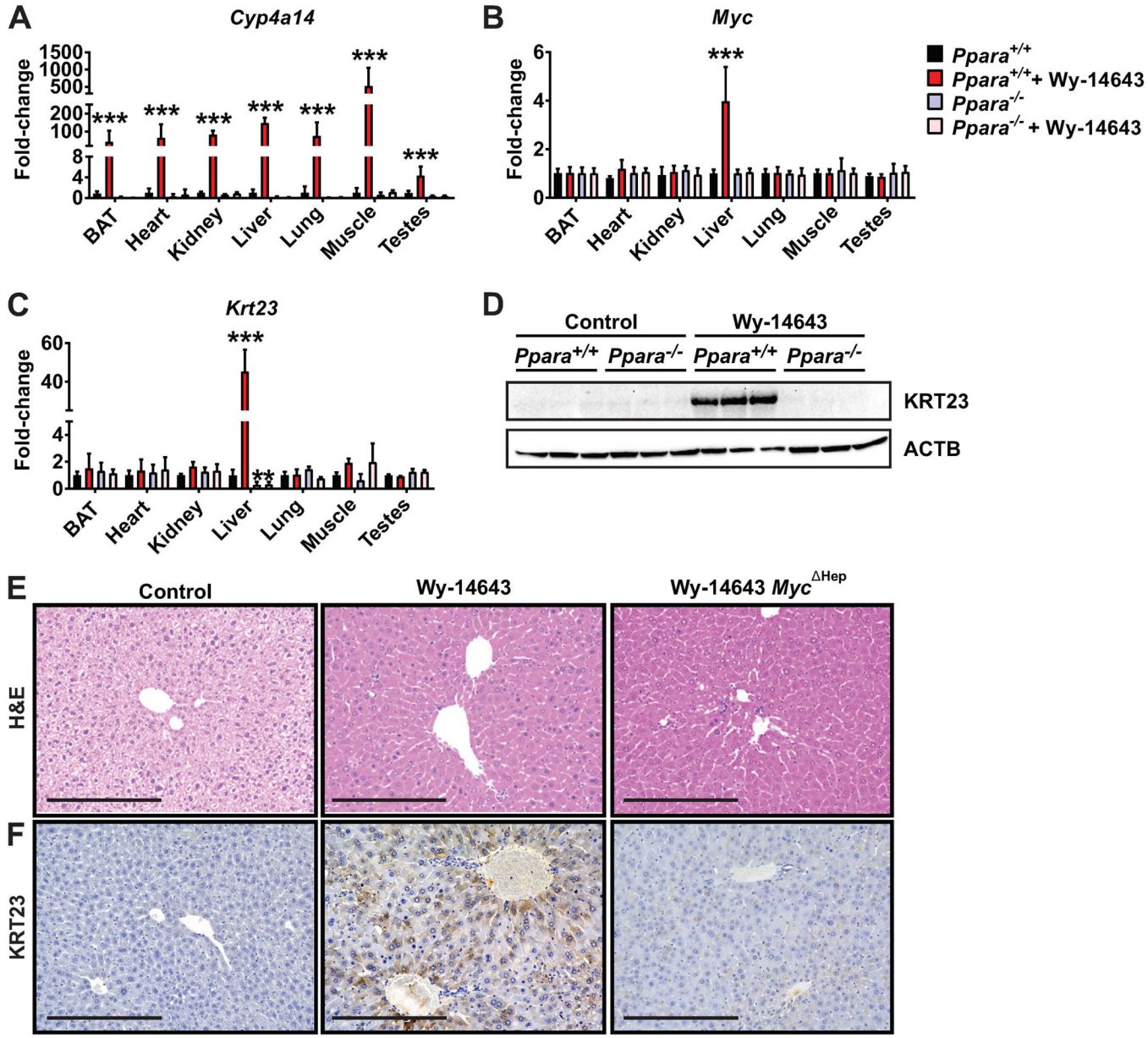

FIG. 2.

Liver-specific KRT23 response supports a role in PPARA-dependent hepatocyte proliferation. Wild-type (*Ppara*$^{+/+}$) and *Ppara*-null (*Ppara*$^{-/-}$) mice were treated with Wy-14643 for 48 hours. (A) qRT-PCR analysis of the PPARA target gene mRNA *Cyp4a14* in different tissues. (B and C) Expression of *Myc* and *Krt23* mRNA in *Ppara*$^{+/+}$ and *Ppara*$^{-/-}$ mice with and without Wy-14643. (D) Western blotting of KRT23 in Wy-14643 treated *Ppara*$^{+/+}$ and *Ppara*$^{-/-}$ mice. (E) Hematoxylin and eosin (H&E) staining of liver sections from control, Wy-14643-treated and Wy-14643 treated in *Myc*$^{\Delta Hep}$ mice. (F) Immunohistochemical staining of KRT23 in liver sections from control, Wy-14643 treated and Wy-14643 treated in *Myc*$^{\Delta Hep}$ mice. Representative images are shown. Scale bars

represent 100 nm (200X). At least five mice were analyzed/genotype and treatment group. Each data point represents the mean ± SD (***, $p < 0.001$).

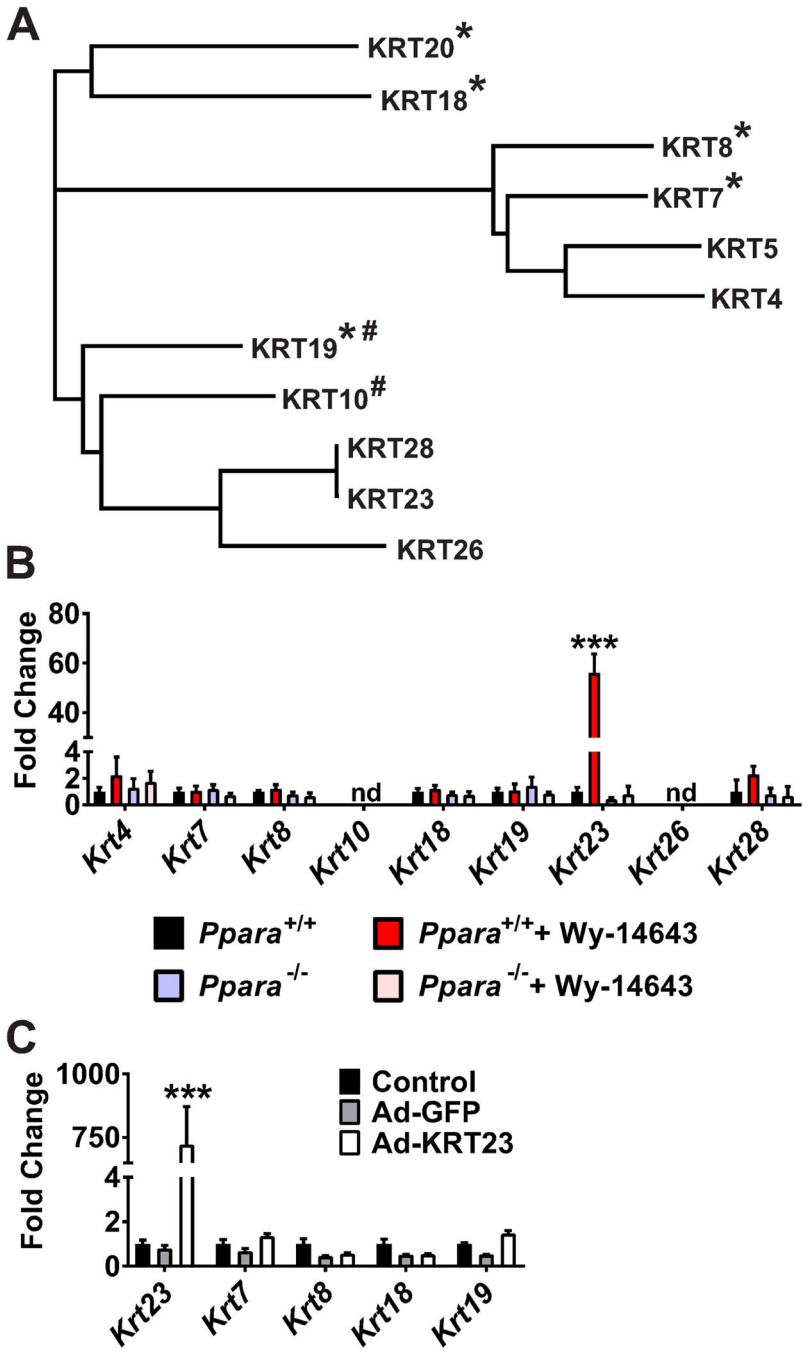

FIG. 3.

KRT23 is the principal keratin upregulated during PPARA-induced hepatocyte proliferation. (A) Dendrogram depicting homology between mouse KRT protein sequences. Diagnostic and prognostic markers denoted by '#' and '*', respectively. (B) *Ppara*$^{+/+}$ and *Ppara*-null mice were treated with Wy-14643 for 48 hours then livers were collected and expression of *Krt* mRNA measured by qRT-PCR. (C) Analysis of *Krt7*, *Krt8*, *Krt18*, *Krt19* and *Krt23* mRNA in primary hepatocytes treated with AAV-KRT23. Experiments were performed on at

least five mice/group. Each data point represents the mean ± SD (nd, not detected; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

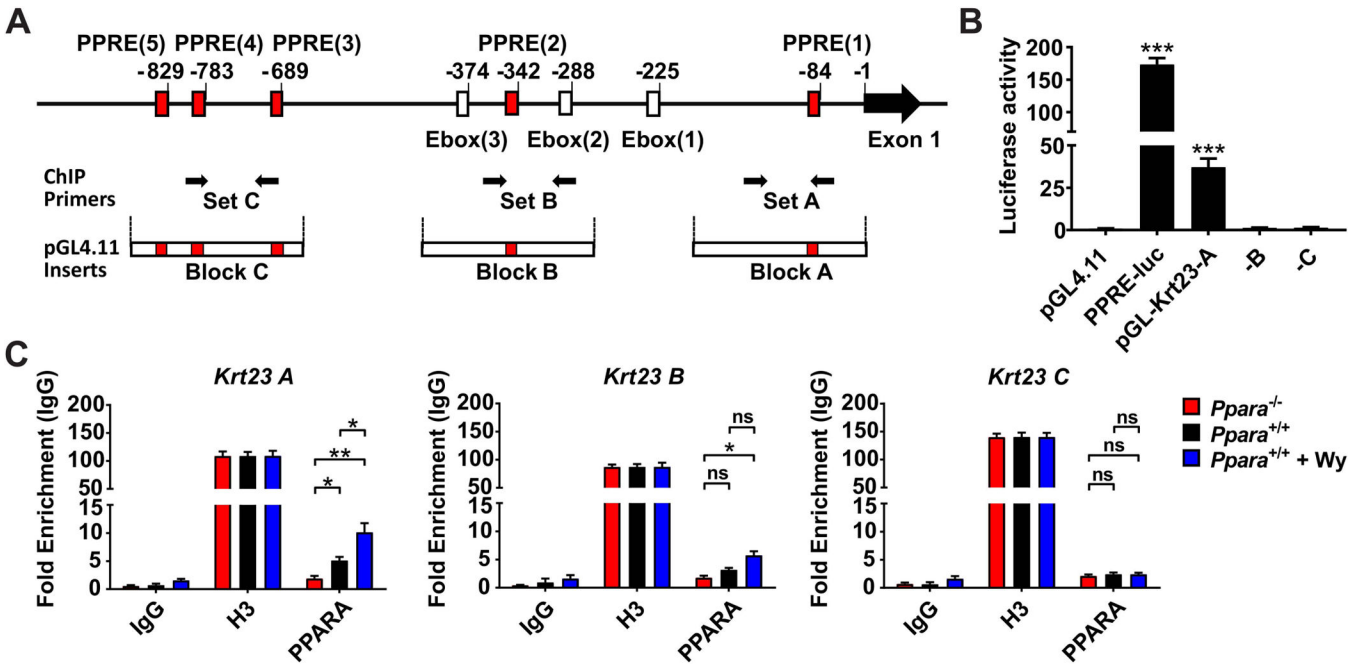

FIG. 4.

PPARA directly regulates *Krt23* through promoter PPRE sites. (A) Schematic representation of predicted PPRE and Ebox sites found within the *Krt23* promoter (−1KB). ChIP primer binding sites and reporter construct inserts are shown below. (B) Luciferase-based reporter assays confirmed functional PPREs sites found within the *Krt23* promoter. (C) PPARA ChIP assays assessed PPRE binding in liver samples from *Ppara*$^{+/+}$ and *Ppara*-null mice treated with Wy-14643. Experiments were performed with at least four replicates. Each data point represents the mean ± SD (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

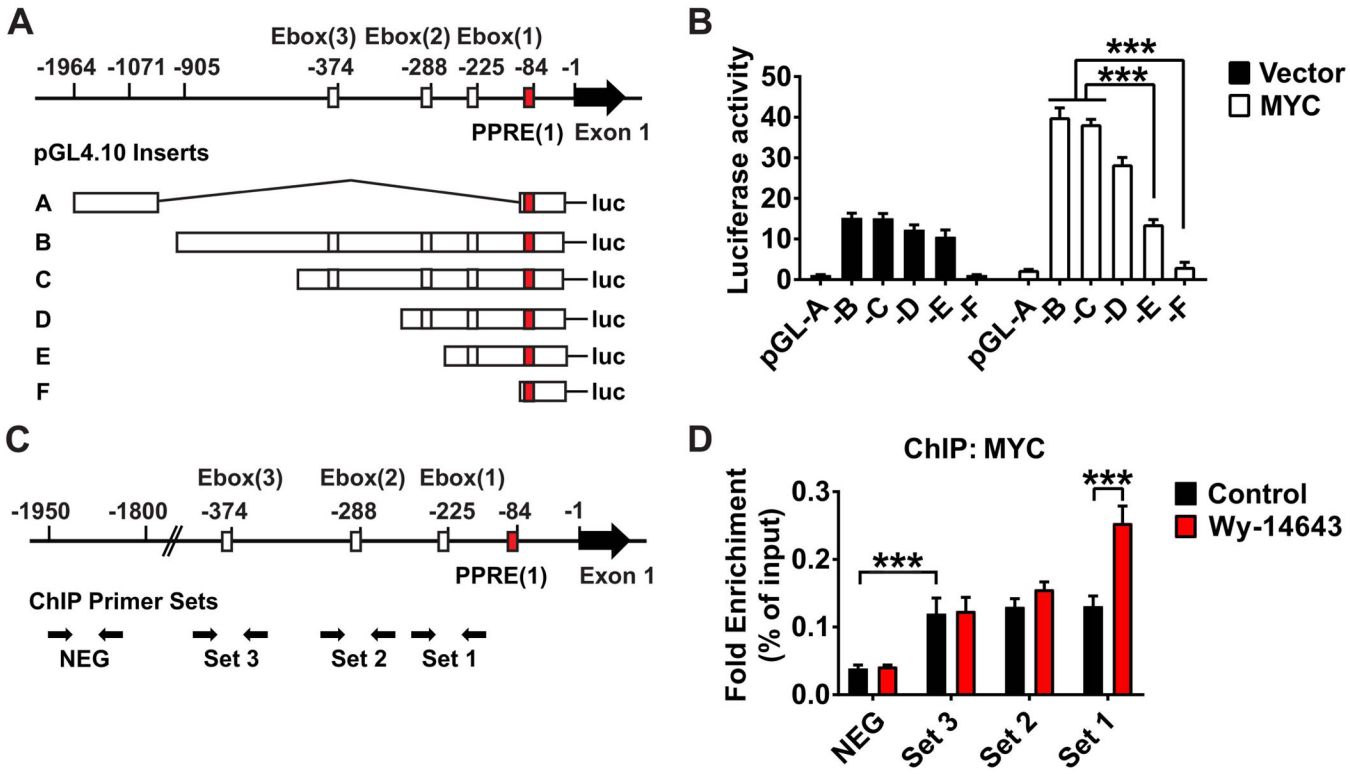

FIG. 5.

MYC directly regulates *Krt23* through promoter Ebox sites. (A) Schematic representation of KRT23 promoter and predicted Ebox binding sites. Reporter construct inserts are detailed below. (B) Luciferase-based reporter assays confirmed MYC transactivation activities in primary hepatocytes transfected with empty plasmid (Vector) or a MYC expression vector (MYC). (C) ChIP primer site locations within the KRT23 promoter used to confirm MYC Ebox binding. (D) MYC ChIP assays for determining promoter occupancy was examined using liver tissue from control and Wy-14643 treated mice. Experiments were performed with at least four replicates. Each data point represents the mean ± SD (***, $p < 0.001$).

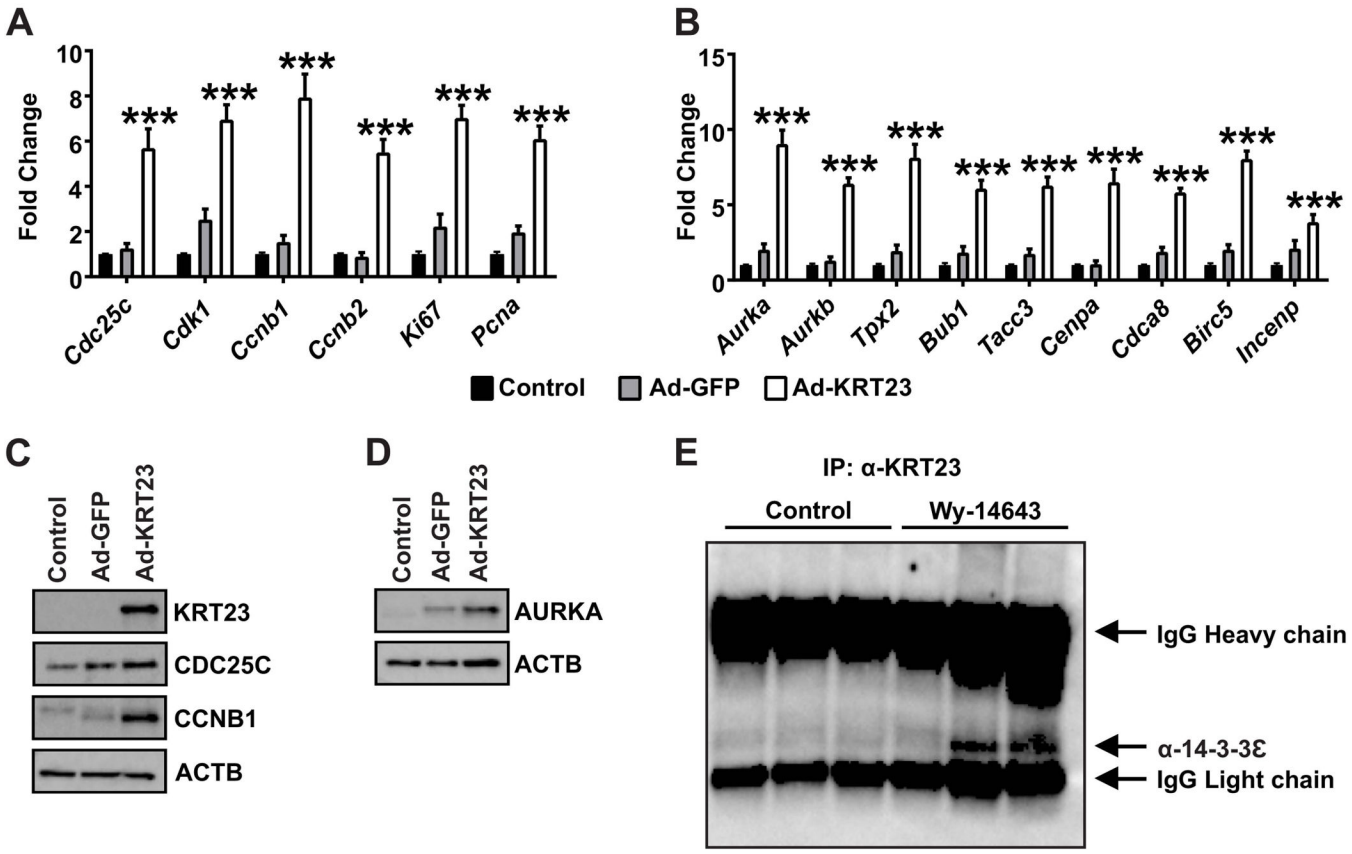

FIG. 6.

Adenovirus expressing KRT23 induces G2/M-related gene expression in primary hepatocytes. (A) qRT-PCR analysis of G2/M-related genes *Cdc25c*, *Cdk1*, *Ccnb1, Ccnb2*, *Ki67,* and *Pcna* mRNA in primary hepatocytes infected with KRT23 expressing adenovirus (Ad-KRT23). Uninfected cells (Control) and cells infected with GFP expressing adenovirus (Ad-GFP) were used as negative controls. (B) Aurora kinase (*Aurk*) and *Aurk*-associated gene expression in KRT23 adenovirus infected primary hepatocytes. (C) Western blotting of KRT23, CDC25C, CCNB, and (D) AURKA protein expression in KRT23 adenovirus infected cells. ACTB expression was used as a loading control. (E) Immunoprecipitation with anti-KRT23 antibody followed by western blotting of 14–3-3ε in liver tissue from control or Wy-14643 treated mice. Each data point represents the mean ± SD (***, $p < 0.001$).

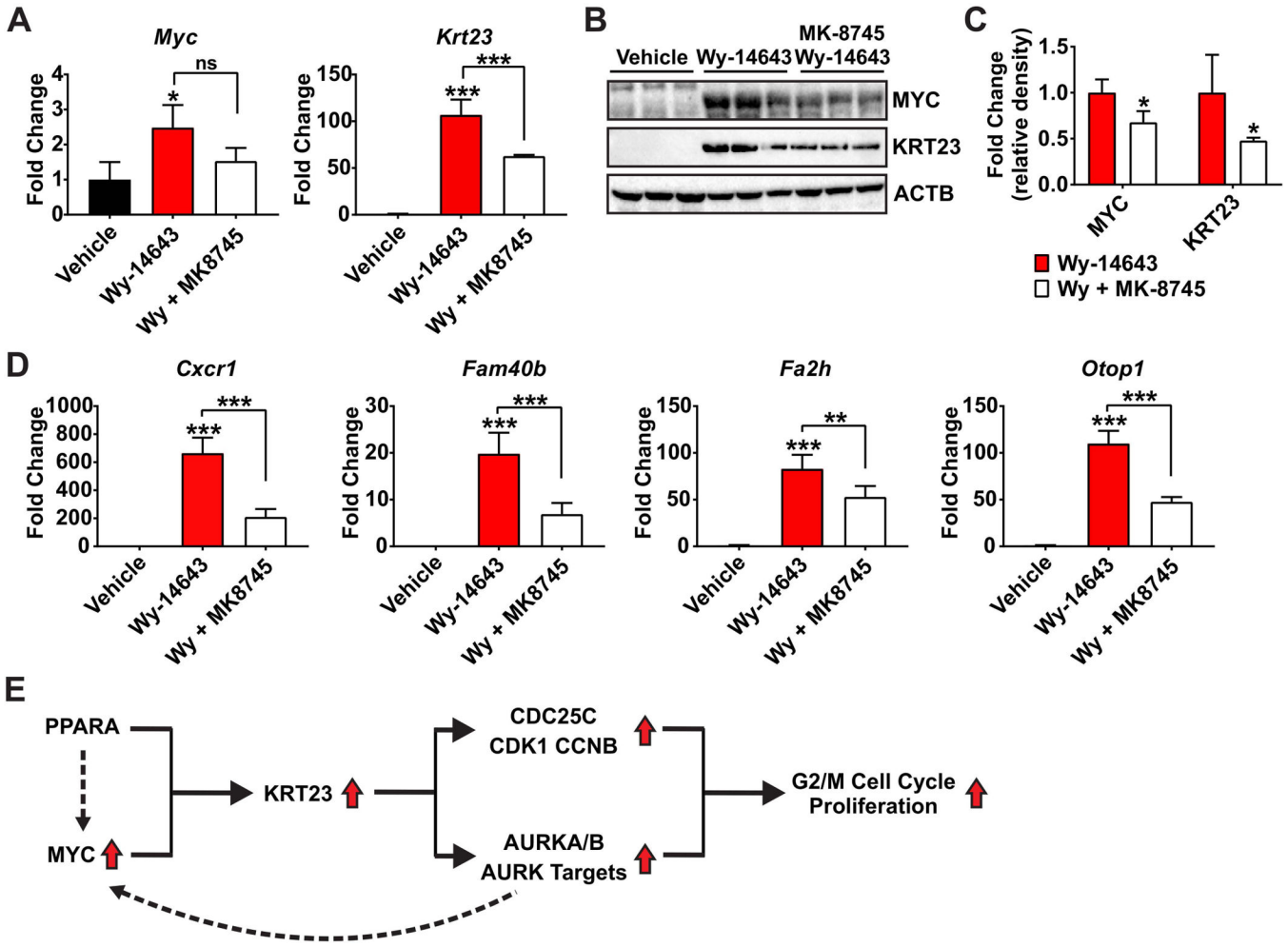

FIG. 7.

Aurora kinase inhibition attenuates MYC and KRT23 expression in the PPARA agonist-induced liver proliferation model. (A) qRT-PCR analysis of *Krt23* and *Myc* mRNA in livers from Wy-14643 treated mice for 3 days or mice treated with Wy-14643 and MK-8745 (Wy + MK-8745) for 3 days. Vehicle treated mice were used as a negative control (Vehicle). (B) Western blot analysis of KRT23 and MYC in liver from Wy-14643 treated mice or treated with MK-8745 in mice. (C) Densitometric analysis of MYC and KRT23 protein expression. (D) Analysis of PPARA-dependent, MYC-amplified gene expression in response to AURK inhibition. (E) Proposed mechanism of action for KRT23 during PPARA-mediated hepatocyte proliferation. Experiments were performed on at least five mice/group. Each data point represents the mean ± SD (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

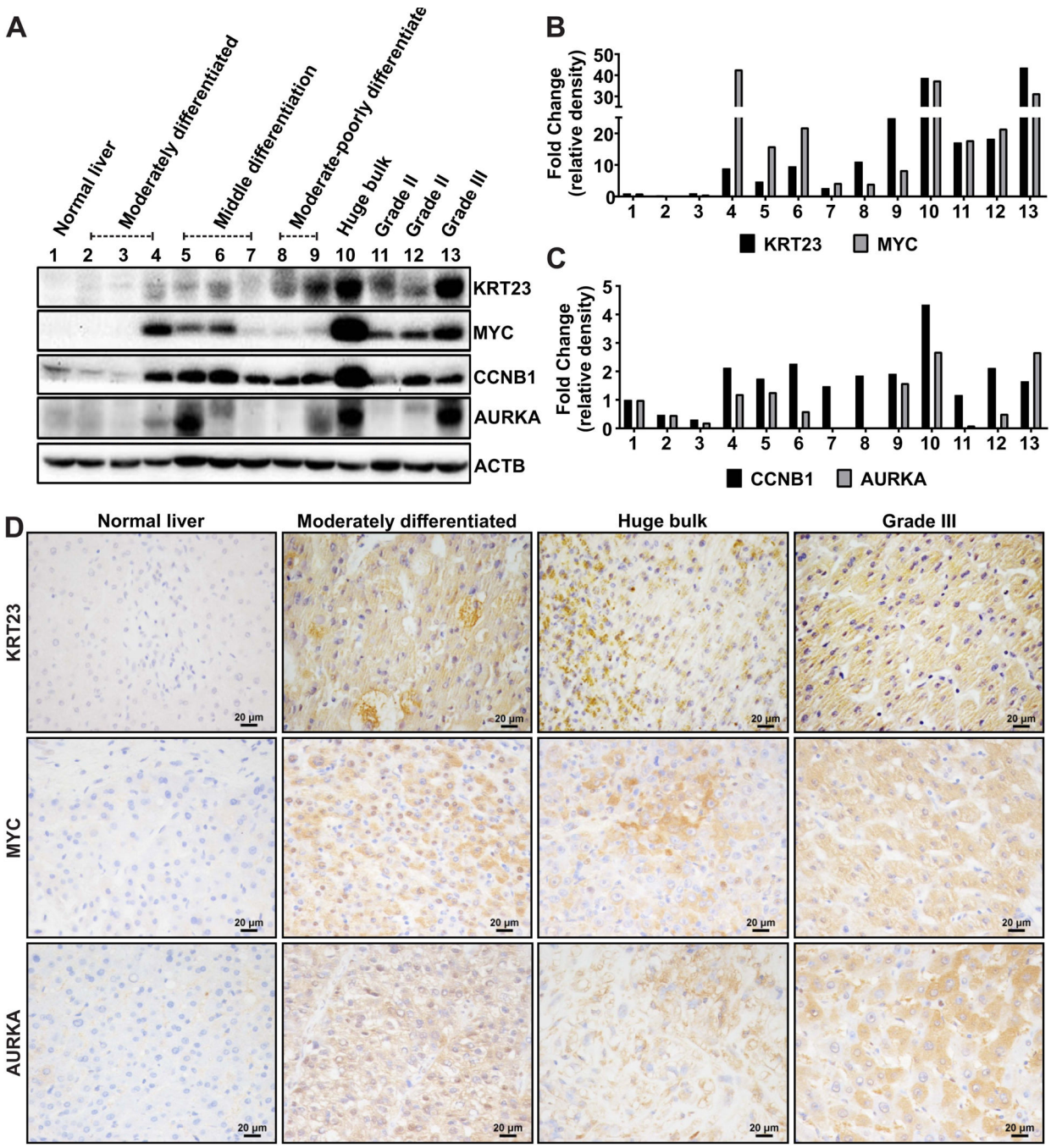

FIG. 8.

KRT23 expression correlates with human HCC progression. **(A)** Western blot analysis of KRT23, MYC, CCNB1, and AURKA protein expression in human liver biopsies. (B) Densitometric analysis of KRT23 and MYC protein expression. (C) Densitometric analysis of CCNB1 and AURKA protein expression. (D) Immunohistochemical staining of KRT23, MYC, and AURKA in from normal or cancerous human liver sections. Representative images are shown. Scale bars represent 20 μm (400X).