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EAG K^+ channel joins the p53−*miR-34*−E2F1 signaling pathway as a terminal effecter component for its oncogenic overexpression and action

Huixian Lin,[1§] Zhe Li,[3§] Xiaobin Luo,[1,2§] Jiening Xiao,[1] Chang Chen,[3] Jianchun Zhang,[1] Deli Dong,[3] Yanjie Lu,[3] Baofeng Yang,[3] and Zhiguo Wang[1,2]*

[1]Research Center, Montreal Heart Institute, Montreal, PQ H1T 1C8 Canada;

[2]Department of Medicine, University of Montreal, Montreal, PQ H3C 3J7, Canada;

[3]Department of Pharmacology (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China), [4]Cardiovascular Research Institute, and [5]Department of Microbiology, Harbin Medical University, Harbin, Heilongjiang 150081, P. R. China

Running Title: EAG K^+ channel in the p53 gene network

[§]The authors with equal contributions

*Correspondence should be addressed to Zhiguo Wang (wangz@mirna-tech.com) or Baofeng Yang (yangbf@ems.hrbmu.edu.cn)

The human *ether-à-go-go*-1 (h-eag1) voltage-dependent K^+ channel is necessary for cell cycle progression and its overexpression stimulates tumorigenesis. We report here that h-eag1 expression is controlled by the p53−*miR-34*−E2F1 pathway through a negative feed-forward mechanism: p53 transactivates *miR-34* that directly represses h-eag1 at post-transcriptional level and indirectly downregulate h-*eag1* at transcriptional level through repressing E2F1. Thus, inhibition of p53 causes oncogenic overexpression of h-eag1 or overexpression of h-eag1 fulfills the oncogenic effect of p53 inactivation.

Abnormally enhanced proliferation often causes loss of control of cell growth leading to tumorigenesis or cancer formation. Several fundamental steps need to be fulfilled at the cellular level for tumorigenesis and these steps can be roughly viewed as characteristic alterations of some physicochemical processes: (1) cell volume, (2) intracellular Ca^{2+}, and (3) intracellular pH. Evidence has emerged indicating a deregulated expression of ion channel protein-coding genes as well as ion channel malfunction as an important step in the development and progression of cancers. The ion channels critically related to cell proliferation and cancer are the K^+ channels[1,2].

Of various categories of K^+ channels, the *ether à go-go* (*eag*) voltage-dependent K^+ channel family stands out the most attractive one in relation to tumor generation, progression and metastasis[1,2]. *Eag1* (or Kv10.1 encoded by *KCNH1*), the founding member of the *eag* family, is restricted in its expression to the brain and placenta in the process of myoblast fusion, indicating that the channel is not normally expressed in differentiated peripheral tissues. On the contrary, *eag1* is expressed in a variety of cell lines derived from human malignant tumors and in clinical samples of several different cancers[3-7], while the surrounding tissues are devoid of *eag1* expression. In these cell lines, *eag1* enhances the proliferation of the cells, and is required for the maintenance of growth. One of the most intriguing aspects of human *eag1* (h-*eag1*) channels is its relationship to cellular transformation; h-*eag1* channels are necessary for progression through the G_1 phase and G_0/G_1 transition of the cell cycle. Cells transfected with h-*eag1* are able to grow in the absence of serum, lose contact inhibition, and induce aggressive tumors when implanted into immune-depressed mice[7]. Moreover, specific inhibition of *eag1* expression by antisense technique[7], siRNA or antibody[8] leads to a reduction in tumor cell proliferation *in vitro* and *in vivo*.

In an initial effort to understand the molecular mechanisms for oncogenic overexpression of *eag1* in cancer cells, we characterized the promoter region of the gene. We used 5'RACE to identify the transcription start site (TSS) which was found located to 152 bp upstream the translation start codon (ATG) of h-*eag1* (GenBank accession No. DQ120124) (**Fig. 1S**). We then defined the minimal promoter

region by luciferase reporter gene assay (**Fig. 2S**). Computer analysis revealed consensus sequences for E2F1, AP2, and SP1 within the core promoter region (position -630/+114), which might act as transactivators of h-*eag1* gene. Using the decoy oligodeoxynucleotide (dODN) approach[9], which contains the perfect binding site for the target transcription factor and can sequestrate the target leading to reduction of transcriptional activity (**Supplementary Materials online**), we revealed a significant role of E2F1, but not of SP1 and AP2, in driving the core promoter activity (**Fig. 1A**). Mutation of the E2F1 *cis*-element rendered a loss of luciferase activity of the core promoter. We further verified E2F1 as a key factor in activating h-*eag1* transcription: E2F1-dODN decreased h-*eag1* mRNA level by ~80% in SHSY5Y human neuroblastoma cells (**Fig. 1B**). With qPCR, we have also ruled out the role of SP1 and AP2 in transcriptional activation of h-*eag1* (**Fig. 1B**). Transfection of E2F1 plasmid, on the other hand, increased h-*eag1* mRNA level by as much as 8 folds, which was diminished by E2F1-dODN. As a negative control, transfection of SP1 plasmid did not significantly alter h-*eag1* mRNA level (**Fig. 1C**) despite that this maneuver was able to enhance expression of h-*erg1* at the mRNA level (**Fig. 1C**), another member of the *eag* K^+ channel gene family, as already established in our previous study[10]. The ability of E2F1 to bind its *cis*-acting elements in the promoter region of h-*eag1* was verified using ChIP and EMSA (**Fig. 1D & 1E**). The transcription factor E2F1 plays a pivotal role in the coordinated expression of genes necessary for cell cycle progression and division, and is known to be an oncoprotein critical for the transcriptional activation of genes that control the rate of tumor cell proliferation[11]. Our finding thus indicates a role of E2F1 in oncogenic upregulation of h-*eag1* expression at the transcriptional level.

Recently, microRNAs (miRNAs) have emerged as a new class of regulators of gene expression. These small non-protein-coding mRNAs primarily elicit repression of protein translation by a partial complementary mechanism with its 5'end 2-8 nts, the "seed site", base-paring the sequence motif(s) in the 3'UTR of target genes. We investigated if h-*eag1* is regulated at the post-transcriptional level by

miRNAs. We first performed computational prediction of h-*eag1* as a target for miRNA regulation. And we identified multiple binding sites for a tumor-suppressor miRNA subfamily *miR-34* (including *miR-34a*, *miR-34b* and *miR-34c*) in the 3'UTR of h-*eag1* mRNA (**Fig. 3S**). To experimentally establish *miR-34*:h-*eag1* interaction, we inserted a fragment of 3'UTR of h-*eag1* containing the *miR-34* target sites into the position downstream the luciferase gene in the pMIR-REPORT^{TM} vector. Transfection of *miR-34a* markedly suppressed the luciferase activities and the effect was reversed by their multiple-target anti-miRNA antisense oligonucleotides (MT-AMO) (**Fig. 1F**; **Supplementary Materials online; Fig. 4S**), a single oligomer capable of targeting all three members of the *miR-34* subfamily[11]. Consistently, *miR-34a* decreased the protein level of h-eag1 by 70% in SHSY5Y cells, as assessed by Western blot analysis, whereas the MT-AMO increased it, presumably through downregulating the endogenous *miR-34a/b/c* (**Fig. 1G**). The same observations were expanded to *miR-34b* and *miR-34c* (**Fig. 5S**). Intriguingly, reduction of h-*eag1* expression was also seen at the mRNA level (**Fig. 1H**). As a negative control, *miR-1* did not cause any appreciable effects on h-*eag1* expression.

It has been documented that *miR-34a* directly targets the mRNA encoding E2F1 and significantly reduces the levels of E2F1 and E2F3 proteins[12,13]. Furthermore, *miR-34a* expression increases during retinoic acid induced differentiation, whereas E2F1 protein levels decrease[12]. We confirmed that transfection of *miR-34a* reduced E2F1 protein levels by ~68% in SHSY5Y cells (**Fig. 1I**) and the same results were obtained with *miR-34b* and *miR-34c* (**Fig. 5S** & **6S**). Moreover, application of the MT-AMO caused significant increases in the protein levels of E2F1 (**Fig. 1I**) and h-eag1 (**Fig. 1G**). These results indicate that *miR-34* regulates h-*eag1* expression through at least two mechanisms. First, *miR-34* directly represses h-eag1 protein. Second, *miR-34* represses E2F1 protein, leading to reduced transcription of h-*eag1*. This latter effect also explains the effectiveness of *miR-34* to decrease h-*eag1* mRNA. This is supported by the experiments showing the lack of effects of *miR-34a* on h-*eag1*

transcript level in cells co-transfected with the E2F1-carrying vector that doe not contain the 3'UTR of E2F1 gene (**Fig. 1J**).

miR-34 has been known to be a direct transcriptional target of p53[13,14] and to mediate the apoptotic action of p53[15]. Thus, changes of p53 activity are deemed to change the level of *miR-34* thereby those of E2F1 and h-eag1 as well. Indeed, p53 activation by Mdm2 inhibitor nutlin-3 (1 μM) increased *miR-34* level (**Fig. 2A**), and simultaneously decreased E2F1 and h-eag1 protein levels (**Fig. 2B**) and h-*eag1* mRNA concentration (**Fig. 2A**). These changes were abrogated when the MT-AMO was co-applied with Nutlin-3. Pifithrin-alpha (PFT-α; 30 μM), the p53 inhibitor, produced exactly the opposite effects to p53 activator. Evidently, p53 negatively regulates expression of h-*eag1*. Moreover, in the presence of p53 inhibitor, exogenously applied *miR-34a* retained the full ability to downregulate E2F1 (**Fig. 2C** & **2D**) and h-eag1 (**Fig. 2E** & **2F**), suggesting that *miR-34* mediates the regulatory role of p53 on E2F1 and h-eag1. Furthermore, in the presence of both p53 inhibitor and the MT-AMO, h-*eag1* expression was markedly upregulated at both mRNA and protein levels, but the E2F1-dODN abolished these increases (**Fig. 2G** & **2H**). On the other hand, the downregulation of h-*eag1* expression induced by co-application of p53 activator and *miR-34a* was abolished by E2F1 overexpression (**Fig. 2I** & **2J**).

Together with the results described above, it is clear that h-eag1 is a terminal effecter component in the p53−*miR-34*−E2F1 pathway for expression regulation and functional signaling. When p53 activity increases in response to environmental and cellular stresses, *miR-34* is deemed to increase, and the increased *miR-34* will decrease E2F1 to diminish h-*eag1* gene transcription and will also repress h-eag1 protein translation as well; diminishment of h-eag1 expression and function then results in a shut-down of cell proliferation or a cell cycle arrest. This implies that h-eag1 executes the cell-cycle checkpoint signal from p53 transmitting along the p53−*miR-34*−E2F1−h-eag1 pathway (**Fig. 7S**). Indeed, expression of h-*eag1* is cell cycle-related; upon synchronization of the cells in G_1 phase with retinoic

acid, eag current amplitude decreased to less than 5% of the control[16]. And retinoic acid has been showed to stimulate expression of *miR-34a* accompanied by a decrease in E2F1 protein level[12].

Next, we sought to examine this notion with the following approaches. We first demonstrated that activation of p53 induced a cell growth arrest in SHSY5Y cells, and overexpression of E2F1 alleviated the cell growth inhibition and so did transfection with the MT-AMO to knock down *miR-34* (**Fig. 3A**). We then observed that the direct growth-stimulating effect of E2F1 was remarkably attenuated by inhibition of h-*eag1* with the antisense oligodeoxynucleotides directed against h-*eag1* gene but not by the sense oligomer for negative control (**Fig. 3B**).

The human eag-related K^+ channel h-erg1 has also been frequently implicated in tumorigenesis[17,18]. To see if h-erg1 is also a part of the p53 network, we analyzed the promoter region that we identified previously[10] and the 3'UTR of the h-*erg1* gene. We found a nearly perfect putative E2F *cis*-element within the core promoter region (**Fig. 8S**) and multiple binding sites for *miR-34a*, *miR-34b* and *miR-34c* in the 3'UTR of the h-*erg1* mRNA (**Fig. 3S**). We further established the role of E2F1 in stimulating h-*erg1* transcription and the role of *miR-34* in repressing h-erg1 translation (**Fig. 9S**). These findings indicate that in addition to h-eag1, h-erg1 is also a component of the p53−*miR-34*−E2F1 pathway.

The tumor-suppressor gene p53 and its downstream genes consist of a complex molecular signaling network and p53 is at the center of this network regulating diverse physiological responses to cancer-related stresses. Activated p53 in response to DNA damage or oncogene activation induces cell cycle arrest, which can be transient or permanent (senescence), or promotes apoptosis in cases where the damage is too severe; conversely, inactivation of p53 causes oncogenic cell growth. Our study herein revealed that *miR-34*, a known transcriptional target of p53, is an important negative regulator of h-eag1 through dual mechanisms by direct repression at the post-transcriptional level and indirect silencing at the transcriptional level via post-transcriptionally repressing E2F1 that we have established to be a transactivator of h-*eag1*. p53 activates *miR-34* transcription; upregulation of *miR-34* represses E2F1 and

h-eag1; repression of E2F1 downregulates expression of h-*eag1*. Therefore, p53 negatively regulates h-eag1 expression by a negative feed-forward mechanism through the p53−*miR-34*−E2F1 pathway and inactivation of p53 activity as it is the case in many cancers can thus cause oncogenic overexpression of h-eag1 by relieving the negative feed-forward regulation. These findings not only help us understand the molecular mechanisms for oncogenic overexpression of h-eag1 in tumorigenesis but also uncover a new layer of mechanisms underlying cell-cycle regulation by the p53−*miR-34*−E2F1−h-eag1 pathway. Moreover, these findings place h-eag1 in the p53−*miR-34*−E2F1−h-eag1 pathway with h-eag as a terminal effecter component and with *miR-34* (and E2F1) as a linker between p53 and h-eag1. Our study therefore fills a gap between p53 pathway and its cellular function mediated by h-eag1.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

Z.W. generated the idea, supervised the studies and wrote the manuscript. B.Y. supervised the studies. H.H., Z.L., and X.L. were the primary investigators of the work, undertaking all aspects of the experiments and data analyses. J.X., C.C., J.Z., D.D. and Y.L. performed some experiments involving Western blot, real-time RT-PCR, and cell growth.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Figure Legends

Figure 1. E2F1 as a transactivator of h-eag1 and *miR-34* as a post-transcriptional repressor of E2F1 and h-eag1. (**A**) Role of E2F1 in driving the h-*eag1* core promoter activity in SHSY5Y human neuroblastoma cells. pGL3-Base: h-*eag1* promoter-free pGL3 vector for control; pGL3-Core: pGL3 vector carrying the h-*eag1* core promoter (a fragment spanning -630/+114); E2F1-dODN, SP1-dODN, and AP2-dODN: the decoy oligodeoxynucleotides targeting E2F1, SP1, and AP2 transcription factors, respectively, co-transfected with pGL3-Core; pGL3-Mutant: pGL3 vector carrying a mutated h-*eag1* core promoter (see **Supplementary Materials online**). Transfection was carried out using lipofectamine 2000. **p*<0.05 *vs* pGL3-Core; n=5 for each group. (**B**) Changes of h-*eag1* mRNA level determined by real-time quantitative RT-PCR (qPCR) in SHSY5Y cells. E2F1-dODN, E2F1-MT dODN, SP1-dODN, or AP2-dODN was transfected alone. Ctl/Lipo: cells mock-treated with lipofectamine 2000; E2F1-MT dODN: the decoy oligodeoxynucleotides targeting E2F1 with mutation at the core region. **p*<0.05 *vs* Ctl/Lipo; n=5 for each group. (**C**) Increase in h-*eag1* mRNA level by overexpression of E2F1 in SHSY5Y cells transfected with the plasmid expressing the E2F1 gene. E2F1-P: the plasmid carrying the E2F1 cDNA. **p*<0.05 *vs* Ctl/Lipo; n=5 for each group. (**D**) Chromatin immunoprecipitation assay (ChIP) to indicate the *in vivo* physical binding of E2F1 to its *cis*-acting elements in the h-*eag1* promoter region in SHSY5Y cells. AB-EAG: antibody to human h-eag1 protein; Input: addition of human genomic DNA. (**E**) Electrophoresis mobility shift assay (EMSA) to indicate the *in vitro* physical binding of E2F1 to the *cis*-elements in the h-*eag1* promoter region using the nuclear extract from SHSY5Y cells. (**F**) Repression of h-eag1 expression by *miR-34a* or *miR-34c*, as reported by luciferase activity assay with the pMIR-REPORT^{TM} luciferase miRNA expression reporter vector carrying the h-*eag1* 3'UTR in HEK293 cells. Ctl: cells transfected with the luciferase vector alone; MT-AMO: the multiple-target anti-miRNA antisense oligonucleotides to *miR-34a*, *miR-34b* and *miR-34c*, co-transfected with the luciferase vector and *miR-34a* or *miR-34c*. **p*<0.05 *vs* Ctl/Lipo; □*p*<0.05 *vs miR-34a*; n=4 for each group. (**G**) Western blot analysis revealing repression of h-eag1 protein by *miR-34a* and *miR-34c* in SHSY5Y cells.

*p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs miR-34a* alone; n=4 for each group. (**H**) Effect of *miR-34* on h-*eag1* mRNA level in SHSY5Y cells. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs miR-34a* alone; n=4 for each group. (**I**) Effect of *miR-34a* on E2F1 protein levels in SHSY5Y cells. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs miR-34a* alone; n=6 for each group. (**J**) Overexpression of h-*eag1* mRNA after transfection of the plasmid expressing the E2F1 cDNA, which was unaffected by *miR-34*. *p<0.05 *vs* Ctl/Lipo; n=4 for each group.

Figure 2. Anti-correlation between p53 activity and h-eag expression. (**A**) & (**B**) Effects of p53 activation by Mdm2 inhibitor nutlin-3 (1 μM) on expression of *miR-34*, E2F1 and h-*eag1* at mRNA and protein levels. SHSY5Y cells were pretreated with nutlin-3 and then transfected with MT-AMO. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs* Nutlin-3 alone; n=4 for each group. (**C**) & (**D**) Downregulation of E2F1 at both mRNA and protein levels by *miR-34a* in the presence of p53 inhibitor Pifithrin-alpha (PFT-α; 30 μM). SHSY5Y cells were pretreated with PFT and then transfected with *miR-34a*. MT-AMO: an antisense oligomer to *miR-34a*, *miR-34b* and *miR-34c*; miR+AMO: co-transfection of *miR-34a* and MT-AMO; NC-miR: scrambled negative control miRNA. Control cells were mock-treated with lipofectamine 2000. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs miR-34a* alone; n=4 for each group. (**E**) & (**F**) Downregulation of h-eag1 at both mRNA and protein levels by *miR-34a* in the presence of p53 inhibitor Pifithrin-alpha (PFT-α; 30 μM). *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs miR-34a* alone; n=4 for each group. (**G**) & (**H**) Effect of E2F1 inhibition by its decoy oligodeoxynucleotides (E2F1-dODN) on h-*eag* expression at both mRNA and protein levels in the presence of both p53 inhibitor and the MT-AMO. SHSY5Y cells were pretreated with PFT (30 μM) and MT-AMO and then transfected with E2F1-dODN to sequestrate E2F1. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs* PFT+MT-AMO; n=4 for each group. (**I**) & (**J**) Effect of E2F1 overexpression on h-*eag* expression in the presence of both p53 activator and *miR-34a*. SHSY5Y cells were pretreated with nutlin-3 (1 μM) and *miR-34a* and then transfected with the plasmid to overexpress E2F1. *p<0.05 *vs* Ctl/Lipo; □p<0.05 *vs* Nutlin-3+*miR-34a*; n=4 for each group.

Figure 3. Effects of the p53−*miR-34*−E2F1−h-eag1 pathway on cell proliferation. (**A**) & (**B**) Effects of p53 activation, E2F1 overexpression and *miR-34* knockdown on SHSY5Y cell proliferation evaluated with MTT assay (**A**) and by population doubling time (PDT) with flow cytometry methods (**B**). Cells were pretreated with nutlin-3 to activate p53 and then transfected with the plasmid carrying E2F1 cDNA for overexpression (E2F1-P) or MT-AMO to knockdown *miR-34*; control cells (Ctl/Lipo) were mock-treated with lipofectamine 2000. **p*<0.05 *vs* Ctl/Lipo; □*p*<0.05 *vs* Nutlin-3 alone; n=4 for each group. (**C**) & (**D**) Effect of the antisense oligodeoxynucleotides (ASO) directed against h-*eag* gene on SHSY5Y cell growth induced by E2F1 overexpression, evaluated with MTT assay (**C**) and by PDT using flow cytometry methods (**D**). Cells were transfected with E2F1 plasmid alone (E2F1-P) or co-transfected with E2F1 plasmid and ASO (+ASO) or SO (sense oligomer for negative control; +SO). **p*<0.05 *vs* Ctl/Lipo; □*p*<0.05 *vs* E2F1-P alone; n=4 for each group.

Figures

Figure 1

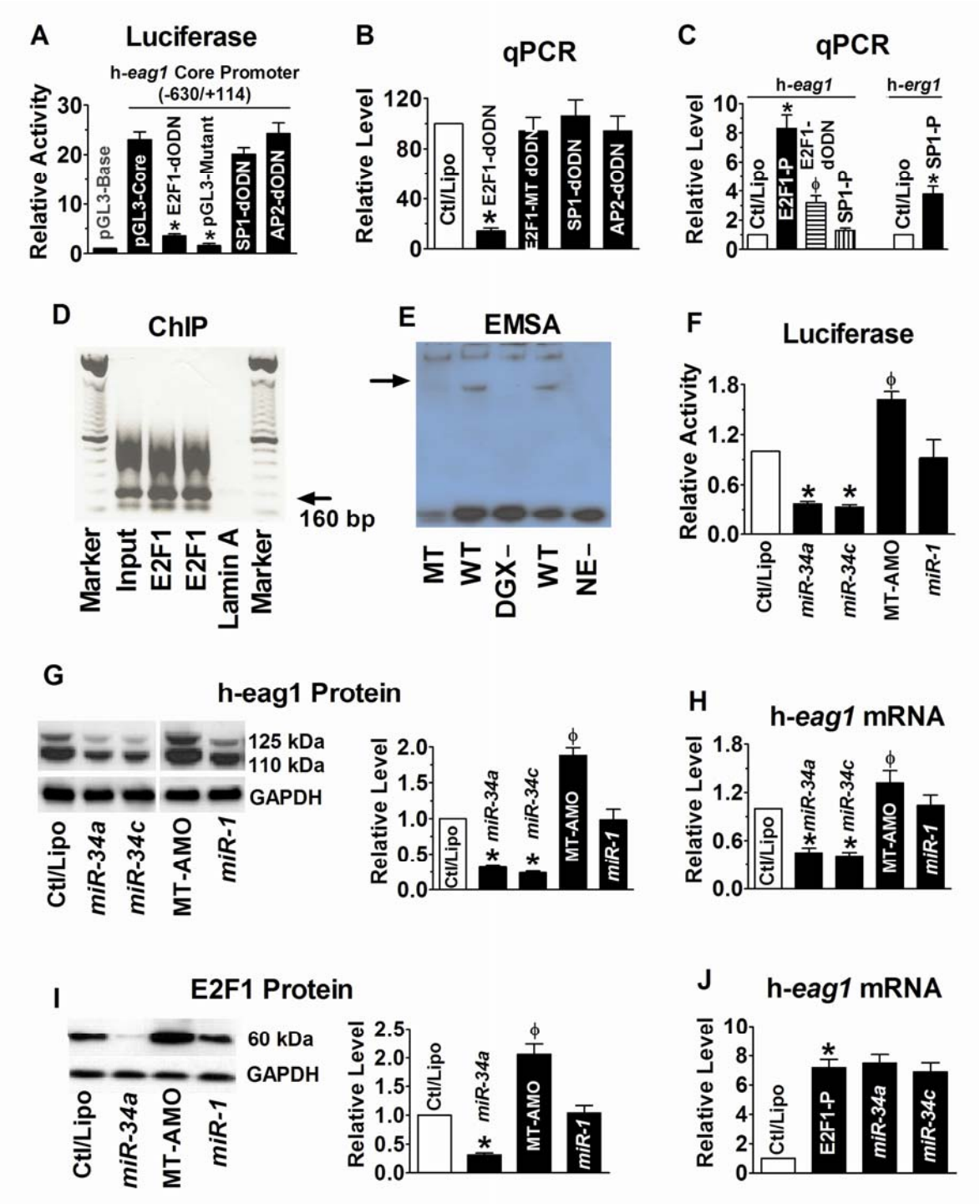

Figure 2

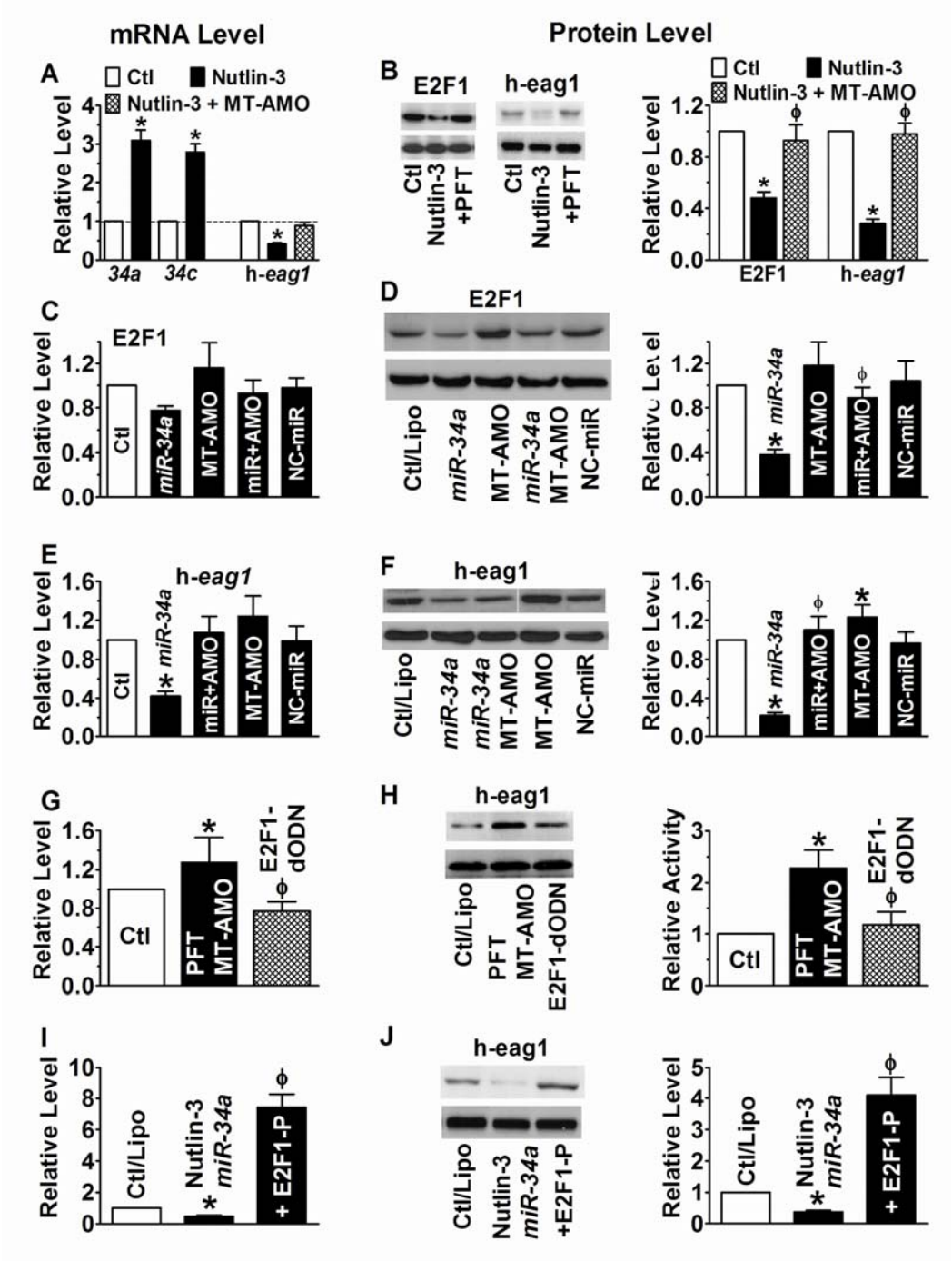

Figure 3