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Interferon regulatory factor-1 is a major regulator of epidermal growth factor receptor gene expression

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Abstract Overexpression of the epidermal growth factor receptor (EGFR) occurs in many tumors and in breast cancer correlates with poor prognosis for treatment. Here, we report that interferon regulatory factor-1 (IRF-1) induces EGFR promoter activity up to 200-fold compared to 3–10-fold induction by other regulators. The region of the promoter that is required for this induction was defined using deletion mutants. In addition, we found that IRF-1 and tricostatin A, a deacetylase inhibitor, have a synergistic effect on EGFR promoter activity. This indicates that the increase in EGFR promoter activity by IRF-1 may also involve changes in chromatin structure. These results identify IRF-1 as a major regulator of EGFR gene expression.
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Key words: Epidermal growth factor receptor; Promoter; Gene regulation; Interferon; DNA binding

1. Introduction

Epidermal growth factor receptor (EGFR) gene expression has to be precisely regulated to insure proper cell growth. Overexpression of EGFR in breast cancer correlates with failure on endocrine therapy [1,2]. The EGFR is considered a marker for cell transformation [3,4], and therefore it has been an attractive target for clinical intervention.

The EGFR promoter contains binding sites for transcription factors, such as SP1, AP2, p53, WT1, and GCF [5–9]. The level of induction or repression by most reported transcription factors ranges between 3- and 10-fold. Many different types of agents have been reported to regulate EGFR gene expression including interferons (IFNs) [10,11]. However, the mechanism by which interferons upregulate EGFR gene expression is unknown. IFNs are known to induce the expression of interferon regulatory factor-1 (IRF-1). IRF-1 is a member of a family of regulatory factors that are highly homologous in their DNA binding domains and bind to the interferon stimulated response element (ISRE) sequences [12–14]. IRF-1 is an important transcription factor that modulates the expression of many of the IFN-inducible genes.

The focus of this study is to determine whether IRF-1 has a major role in modulating EGFR gene expression. Such understanding will help in the search for new approaches to control the expression of EGFR in malignant tumors and in breast cancer in particular.

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2. Materials and methods

2.1. Materials

The human breast cancer cell line MCF-7 was purchased from ATCC. U3A cells were a gift from G. Stark. All cells were propagated in Dulbecco's modified Eagle's medium (Life Technologies) in the presence of 10% fetal calf serum. Luciferase constructs of the EGFR promoter, pERLUC-1, pERLUC-4, pERLUC-5, pERLUC-6 and pERLUC-9, were prepared by ligating *Hin*dIII promoter fragments from pERCAT constructs [5] into pGL-basic (Promega). The IRF-1 expression vector (pAct-1) was a gift from T. Taniguchi. The pBS based plasmid for in vitro transcription/translation was a gift from K. Ozato.

2.2. Luciferase assays

U3A cells grown in triplicate in 35 mm diameter plates were transfected with the appropriate expression vector by lipofectamine (Life Technologies). The EGFR promoter reporter plasmids (500 mg) were cotransfected with the indicated amount of IRF-1 expression vector and with the empty vector LK440. DNA concentration was kept constant by addition of herring sperm DNA. Cells were harvested 24 h after transfection and cell extracts prepared according to the protocol from Analytical Luminescence Laboratory. All luciferase activities were normalized for protein concentration. All experiment were performed in triplicate or more.

2.3. Electrophoretic mobility shift assays

Double-stranded DNA oligonucleotides were used for electrophoretic mobility shift assays (EMSAs). EGFRp-75: GCCGACAAACACAGTTCGGAAATAAGGTTCTCGAAGTGAAAACGCTTC, EGFRp-132: GTGTAACCGAAGTTTCATGGGTACCGACCAACGTTATTTGTAA, and IRF-1-3n: AAGTGAAAGTGAAAGTGAAAGTGA (the IRF-1 binding site [14]). As a non-specific competitor (NS), a 42 bp oligonucleotide GCF2-3x was used.

IRF-1 was generated by coupled in vitro transcription/translation using reticulocyte lysates (Promega). IRF-1 was incubated with double-stranded end-labeled oligonucleotides in a buffer containing 1 μg of poly(dI-dC). The protein-DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel.

2.4. Western blotting

Whole cell extracts (30 μg) were resolved on a 4–12% SDS-PAGE, transferred onto PVDF membrane (Novex), followed by immunoblotting using the ECL detection system as suggested by the manufacturer (Amersham).

3. Results and discussion

To understand what factors modulate the induction of the EGFR by IFNγ, we examined the response to one of the major transcription factors induced by IFNs, IRF-1. Since IRF-1 is known to be induced by STAT-1 [15], the human fibroblast cell line U3A was chosen for this experiment because it lacks the expression of STAT-1 [16]. This enabled us to eliminate possible effects of endogenous STAT-1. To determine the effect of this factor on EGFR promoter activity, we

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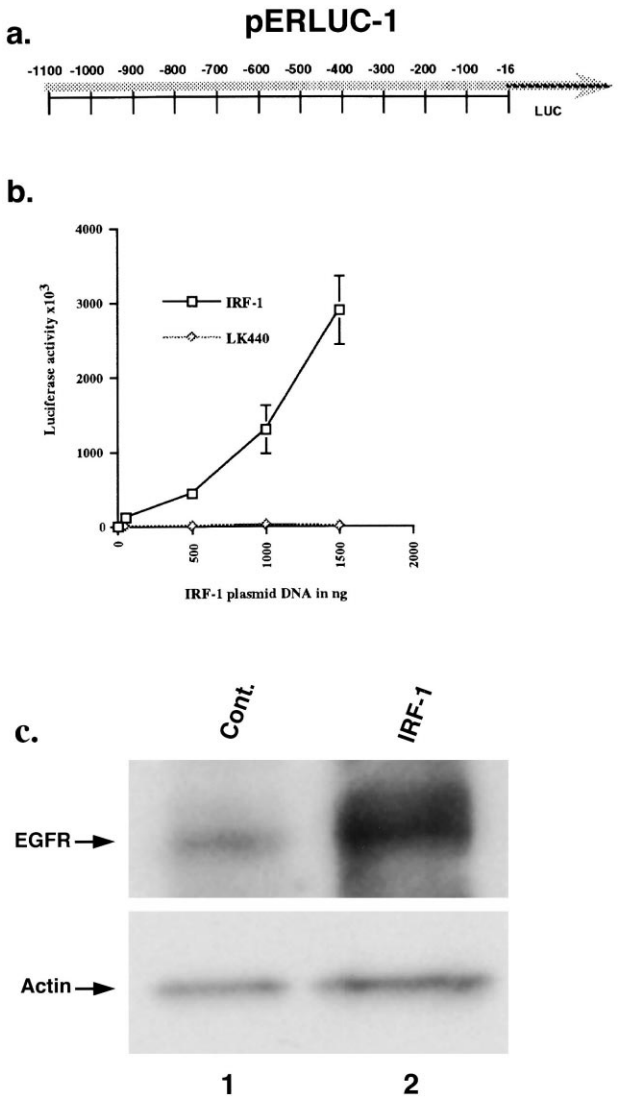

Fig. 1. EGFR gene expression is induced by IRF-1. a: Schematic diagram of pERLUC-1. b: IRF-1 induction of the EGFR promoter. U3A cells ($3–6\times10^5$) were transfected with 0, 50, 500, 1000 and 1500 ng of pAct (IRF-1) or control plasmid LK440, in the presence of 500 ng of pERLUC-1. 24 h post transfection cells were harvested and luciferase activity was measured. □ and ◇ represent the induction of pERLUC-1 by IRF-1 and control vector respectively. c: IRF-1 induction of endogenous EGFR. Subconfluent MCF-7 cells were transfected with 1500 ng of pAct-1 (IRF-1) for 24 h. A total protein extract was prepared and 30 μg was loaded onto a 4–12% SDS-polyacrylamide gel and analysed by Western blotting.

cotransfected the full-length EGFR promoter construct, pERLUC-1 (Fig. 1a) with increasing amounts of the IRF-1 expression vector. This resulted in a dose-dependent increase of up to 200-fold in luciferase activity (Fig. 1b). Such high levels of induction of EGFR promoter activity have not been previously reported.

The dramatic increase of the EGFR promoter activity by IRF-1 prompted us to examine whether IRF-1 could induce the expression of the endogenous EGFR. We selected MCF-7 breast cancer cells to examine this because they have been used as a model system to study the effect of EGF on human breast cancer cells and they express low levels of EGFR [17,18]. MCF-7 cells were transfected with IRF-1 expression vector and the expression of the EGFR was analyzed by Western blotting. As shown in Fig. 1c, there is a 10-fold increase in EGFR expression in cells transfected with IRF-1 expression vector. Our Western analysis of MCF-7 cells confirmed the induction of the EGFR expression by IFNγ (data not shown), as had been previously reported [19,20]. This experiment indicates that IRF-1 can induce endogenous EGFR expression as well as the transfected EGFR promoter activity.

To localize the region of the promoter that is required for the increased activity by IRF-1, deletion mutants of the EGFR promoter were constructed (Fig. 2a). As shown in Fig. 2b, the induced activity of the EGFR promoter decreased dramatically from pERLUC-1 to pERLUC-4, after deletion of 198 nucleotides (Fig. 2b). These results indicated that the region between −1109 and −911 in the EGFR promoter is required for maximal induction by IRF-1. To further analyze the nature of the IRF-1 induction, the sequence of the EGFR promoter between nucleotides −1109 and −911 was analyzed for potential IRF-1 binding sites (Fig. 3a). By comparing this sequence with the IRF-1 and the ISRE consensus binding sequences G(A)AAA$^{G}/_{C}$/$^{T}/_{C}$GAAA$^{G}/_{C}$$^{T}/_{C}$ and $^{A}/_{G}$NGAAANNGAAACT respectively [21], two oligonucleotides encompassing nucleotides −1035 to −989 (EGFRp-75) and −976 to −934 (EGFRp-132) were synthesized and used in EMSAs to determine if IRF-1 could bind to this region (Fig. 3a). As shown in Fig. 3b lane 3, IRF-1 was able to bind to ^{32}P-labeled

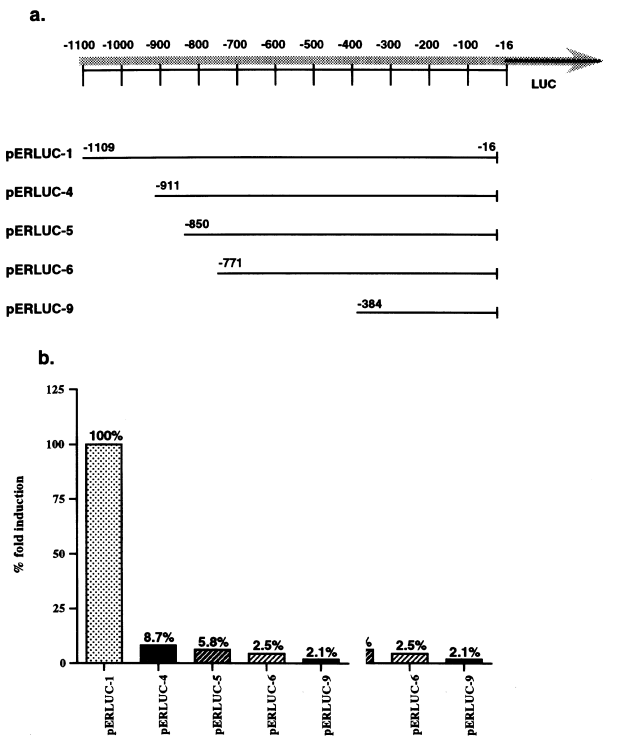

Fig. 2. The 5′ end of the EGFR promoter is required for inducibility by IRF-1. a: Schematic diagram of EGFR reporter constructs. Full-length and deletion mutants of the EGFR promoter were fused to the luciferase gene. b: IRF-1 induction of EGFR promoter deletion mutants. MCF-7 cells ($3–6\times10^5$) were transfected with 500 ng of each of the EGFR promoter construct, pERLUC-1, pERLUC-4, pERLUC-5, pERLUC-6 and pERLUC-9, in the presence of 1500 ng of pAct-1 (IRF-1). The empty vector LK440 was used as a control. Cells were harvested after 24 h and the luciferase activity was measured.

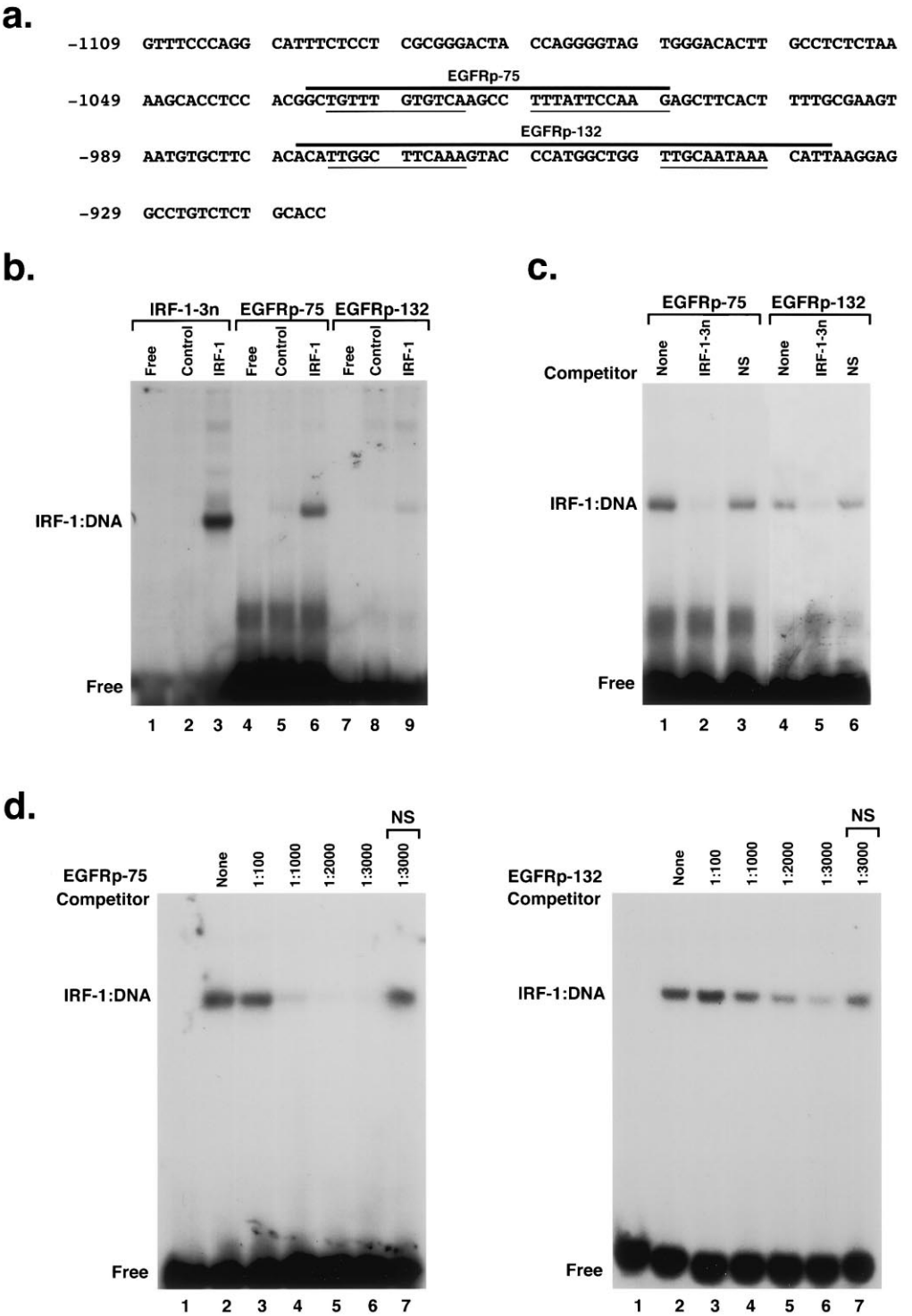

Fig. 3. IRF-1 binds specifically to sequences in the EGFR promoter. a: EGFRp-75 and EGFRp-132 oligonucleotide sequences. EGFRp oligonucleotides were derived from the EGFR promoter, nucleotides −1035 to −989 and nucleotides −976 to −934, respectively. b: EMSAs of IRF-1 binding to EGFRp-75 and EGFRp-132 oligonucleotides. The control for each oligonucleotide was the reticulocyte lysate alone (lanes 2, 5 and 8). c: Competition assays for IRF-1 binding. Unlabeled IRF-1-3n oligonucleotide was used to compete with ^{32}P-labeled EGFRp-75 and EGFRp-132 oligonucleotides using EMSAs. IRF-1 (2 μl) was incubated with labeled EGFRp-75 (lane 1) and EGFRp-132 (lane 4) alone or in combination with a 1:1000 molar ratio of cold IRF-1-3n (lanes 2 and 5). d: Reverse competition analysis. Labeled IRF-1-3n was competed with cold EGFRp-75 (left panel) and EGFRp-132 (right panel) oligonucleotides at increasing molar ratios up to 1:3000 (lanes 3–6) using EMSAs.

IRF-1-3n, an oligonucleotide containing the IRF-1 binding site. EMSAs using EGFRp-75 and EGFRp-132 indicated that although these two EGFRp oligonucleotides do not contain the perfect IRF-1 binding site, IRF-1 binds to these sequences as well (Fig. 3b, lanes 6 and 9, respectively). The complexes IRF-1/EGFRp-75 and IRF-1/EGFRp-132 mi-

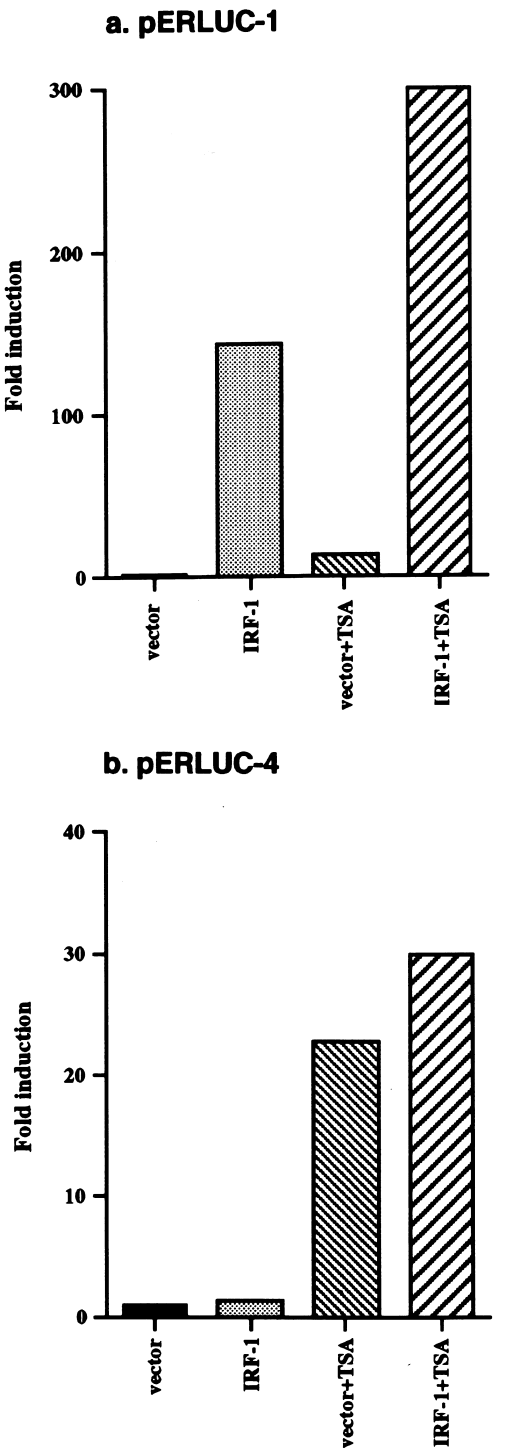

Fig. 4. TSA acts synergistically with IRF-1 to increase the EGFR promoter activity. U3A cells were cotransfected with 1500 ng IRF-1 or empty vector (LK440) and (a) 500 ng full-length EGFR promoter reported construct, pERLUC-1, or (b) 500 ng deletion mutant pERLUC-4. TSA was added at 1 μM 5 h after transfection. Luciferase activity was measured 24 h after transfection.

grated to the expected location relative to the IRF-1/IRF-1-3n complex.

To examine the specificity of the IRF-1 binding to the EGFR promoter, we performed EMSAs with competition. Cold IRF-1-3n and a non-specific oligonucleotide were used to compete with ^{32}P-labeled EGFRp-75 and EGFRp-132 oligonucleotides for IRF-1 binding. IRF-1-3n competed with both EGFRp oligonucleotides (Fig. 3c, lanes 2 and 5) while the non-specific oligonucleotide (NS) at the same molar ratio had no effect on the binding (Fig. 3c, lanes 3 and 6). In a reverse competition assay, EGFR oligonucleotides EGFRp-75 and EGFRp-132 were used as competitors with the ^{32}P-labeled IRF-1-3n. As displayed in Fig. 3d, both oligonucleotides competed with IRF-1-3n in a concentration-dependent manner. To obtain complete competition, a 3000-fold molar excess of EGFRp-132 (right panel, lane 6) and a 1000-fold molar excess of EGFRp-75 (left panel, lane 4) were required. The requirement for this large excess of competitor is unusual but is consistent with previous reports by Tanaka et al. [22]. Although these data show that IRF-1 can bind to DNA elements in the EGFR promoter, IRF-1 can also activate gene transcription through protein-protein interaction with other transcription factors such as HMGI(Y) and NF-κB [23]. The physical interaction of IRF-1 to these transcription factors facilitates its DNA binding activity. In addition, it has been suggested that the IRF family may increase transcription activity by altering chromatin structure [24]. This could be the result of direct binding and recruitment of histone acetyltransferases such as PCAF [25]. Histone acetylation has been shown to be an important process in controlling gene expression [26]. Thus, we examined whether IRF-1 induction of EGFR promoter activity was affected by TSA, a deacetylase inhibitor. U3A cells were cotransfected with IRF-1 and the full-length EGFR promoter construct, pERLUC-1, followed by 1 μM TSA treatment. As illustrated in Fig. 4a, IRF-1 and TSA cooperate in a synergistic fashion to potentiate the induction of the EGFR promoter activity. This indicates that acetylation may be involved in the IRF-1 induction of the EGFR promoter activity. IRF-1 or TSA may induce the acetylation and activation of other factors which interact with IRF-1 to bind the EGFR promoter. There is also an increase in the activity of the control with TSA alone (Fig. 4a, lane 3). This is not surprising since TSA is a general inhibitor of deacetylases. Next, we analyzed whether the region between −1109 and −911, which was found to be required for the IRF-1 induction of the EGFR promoter, was also important for potentiating the IRF-1 induction by TSA. A comparison was made with the deletion mutant, pERLUC-4 (Fig. 4b), which is missing the −1101 to −911 region (Fig. 2a). U3A cells were cotransfected with IRF-1 and pERLUC-1 or with pERLUC-4 followed by TSA treatment. Luciferase analysis showed that although TSA increased the overall activity in the case of pERLUC-4, it could not potentiate the IRF-1 induction of the EGFR promoter to the same levels obtained with pERLUC-1 (Fig. 4b).

These results suggest that IRF-1 may mediate the IFN induction of EGFR expression since IRF-1 is a major transcription factor that is induced by IFNs. On the other hand, the induction of EGFR expression by IRF-1 may be independent of IFN. IRF-1 can be induced by other factors such as TNF-α, IL-1, IL-6, ILF, and prolactin [14,27–30]. Until now it was not clear whether there are factors that have large modulating activities on this gene. The significance of this report is twofold. First, we propose a pathway for the upregulation of EGFR by IFN that is mediated by IRF-1. Secondly, we identified IRF-1 as a major regulator of the EGFR promoter

which can potentially be targeted for therapeutic intervention to control EGFR expression.

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