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J Cell Biochem. Author manuscript; available in PMC 2011 September 1.

Published in final edited form as:
J Cell Biochem. 2010 September 1; 111(1): 207–217. doi:10.1002/jcb.22690.

EGR1, EGR2, and EGR3 activate the expression of their coregulator NAB2 establishing a negative feedback loop in cells of neuroectodermal and epithelial origin

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Abstract

The inducible zinc finger transcription factors EGR1, EGR2, and EGR3 regulate the expression of numerous genes involved in differentiation, growth, and response to extracellular signals. Their activity is modulated in part through NAB2 which is induced by the same stimuli. In melanoma and carcinoma cells EGR1 activates *NAB2* expression. In T lymphocytes EGR2 and EGR3 have been shown to inhibit NAB2 expression. Therefore, we investigated the influence of EGR2 and EGR3 on NAB2 expression in melanoma and carcinoma cells. Here we show that like EGR1, EGR2 and EGR3 induce *NAB2* expression in these cells. EGR1 and EGR3 act in concert on the *NAB2* promoter and are more potent activators of *NAB2* transcription than EGR2. EGR1-, EGR2-, and EGR3-induced *NAB2* promoter activity is mediated through similar cis-regulatory elements and the activation by each EGR is repressed by NAB2. Kinetic studies suggest that induction of EGR1 leads to low *NAB2* expression while EGR2 and EGR3 are necessary for maximal and sustained expression. As aleady shown for *EGR1*, reduction of *EGR2* or *EGR3* expression by siRNAs reduced endogenous *NAB2* levels. Depletion of *EGR3* also resulted in a reduction of *EGR2* levels confirming *EGR2* as a target gene of EGR3. Our results suggest that in many cells of neuroectodermal and epithelial origin EGR1, EGR2, and EGR3 activate *NAB2* transcription which is in turn is repressed by NAB2, thus establishing a negative feedback loop. This points to a complex relationship between the EGR factors and NAB2 expression likely depending on the cellular context.

Keywords

NAB2; early growth response; EGR; transcription factor; negative feedback loop; autoregulation; promoter

The zinc finger transcription factors of the early growth response family (EGR[3]) are immediate early response genes that are important for the induction of cellular programs of differentiation, proliferation, and cell death in response to environmental stimuli (Carter et al., 2007;Thiel et al., 2002;Unoki et al., 2003). The most prominent members of the EGR

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[3]The abbreviations used are: EGR, early growth response; NGFI-A, nerve growth factor-induced protein A; Nab, NGFI-A binding protein; MADER, melanoma associated delayed early response gene; SP1, specificity protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; AP2, activator protein 2; CREB, cAMP-responsive element binding protein; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TCR, T cell receptor; siRNA, short-interfering RNA; PMA, phorbol myristate acetate; EMSA, electrophoretic mobility shift assay; PHLDA1, pleckstrin homology-like domain A-1; EGF, epidermal growth factor.

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family are EGR1, EGR2, and EGR3, which share consensus DNA binding sequences. While the expression of the EGR family members is low or undetectable in resting cells, it is rapidly and transiently induced by a wide variety of extracellular signals such as cytokines, growth factors, T cell receptor (TCR) activation, and toxic substances (Beckmann et al., 1997;Collins et al., 2006;Collins et al., 2008;Honkaniemi et al., 2000;Skerka et al., 1997). Target genes include growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (Khachigian et al., 1995;Liu et al., 2003), as well as genes involved in the differentiation of hematopoietic cells (Collins et al., 2008;Laslo et al., 2006) and in neuronal development (Mager et al., 2008;Nagarajan et al., 2001). The activity of the EGR transcription factors is controlled in part through the members of the NGFI-A binding protein (NAB) family, namely NAB1 and NAB2, which modulate the activity of the EGR family members by binding to them (Russo et al., 1995;Sevetson et al., 2000;Svaren et al., 1996;Svaren et al., 1998). Nab proteins were first identified as Egr1 binding proteins in a two hybrid assay (Russo et al., 1995;Svaren et al., 1996). NAB2, which is also known as melanoma associated delayed early response gene (MADER), was independently identified as a nuclear protein overexpressed in human malignant melanoma (Kirsch et al., 1996). NAB2 can inhibit EGR1, EGR2, and EGR3, whereas NAB1 is able to modulate the activity of only EGR1 and EGR2. Furthermore, NAB1 is constitutively expressed in most cell types, while NAB2 is a delayed early response gene and is induced by the same environmental signals that lead to EGR expression. This implies that NAB2 is the more important regulator of the EGR family, crucial for controlling the EGR-mediated activation of transcription.

Although Nab2 was originally defined as a corepressor of the EGR transcription factors (Svaren et al., 1996), recent studies showed that NAB2 can act also as a coactivator of EGR-mediated gene transcription (Collins et al., 2006; Sevetson et al., 2000). In addition, the influence of the EGR members on NAB2 expression and whether they are coactivated or corepressed by NAB2 seems to be highly dependent on the cellular context. During the determination of hematopoietic cell fates EGR1 and EGR2 functions are redundant in promoting macrophage differentiation by limiting growth factor independent 1 (Gfi-1) transcription repressor function (Laslo et al., 2006). Neither EGR2 or NAB2 alone influence Gfi-1 expression, but together they repress the Gfi-1 gene, resulting in the inhibition of the neutrophil program. TCR-induced NAB2 and EGR1 act together to activate the interleukin-2 promoter leading to T cell activation (Collins et al., 2006). However, TCR-induced EGR2 and EGR3 inhibit T cell activation by repressing the expression of NAB2 and EGR1 (Collins et al., 2008).

Recently, EGR1 was shown to be a major positive regulator of the transcription of its own repressor *NAB2* in melanoma and carcinoma cells (Kumbrink et al., 2005). The EGR1-mediated activation of *NAB2* transcription was repressed by NAB2, indicating the existence of an EGR1/NAB2 negative feedback mechanism. Electromobility shift assays (EMSAs) pointed to the participation of other members of the EGR family in the regulation of NAB2 expression. In the studies presented here we show that in tumor cells of neuroectodermal and epithelial origin EGR1, EGR2, and EGR3 act as positive regulators of *NAB2* gene expression. As shown previously for EGR1 reduction of either *EGR2* or *EGR3* by short-interfering (si) RNAs reduced *NAB2* expression. Expression kinetics in response to phorbol esters suggest that EGR2 and EGR3 are necessary for the maximum induction and maintenance of *NAB2* expression. The EGR-mediated activation of *NAB2* transcription was repressed by NAB2, further supporting the existence of an EGR/NAB2 negative feedback mechanism in cells of neuroectodermal and epithelial origin which prevents continual NAB2-mediated modulation of EGR target genes in response to extracellular signals.

MATERIAL & METHODS

Cell Lines and culture conditions

Cell lines were obtained from the ATCC (Manassas, VA), established in our laboratory, or obtained through exchange. Cell lines were cultured in RPMI 1640 Medium (Biochrom AG, Berlin, Germany) supplemented with 5% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO_2. Cell lines used were, human melanoma: Mel 888, Mel Ei, Mel Juso, and SK-MEL-23; human colon carcinoma: Colo 320DM and LoVo; human breast carcinoma: MCF-7. Cells were seeded 16 hours prior to stimulation by the addition of phorbol myristate acetate (PMA, final concentration 10 ng/ml) obtained from Sigma-Aldrich (St. Louis, MO, USA) for indicated times. All cells were routinely tested for mycoplasma contamination.

Generation of expression constructs

The generation of the expression constructs for human EGR1, NAB1, NAB2, and pleckstrin homology-like domain A-1 (PHLDA1) was described previously (Kumbrink et al., 2005; Neef et al., 2002). Dominant negative (dn)NAB2 was generated by a single nucleotide exchange resulting in amino acid change E51K using the *QuikChange™ XL site-directed mutagenesis kit* (Stratagene, La Jolla, CA, USA). Wild type *NAB2* served as template and primers were: 5′-GGGTGAGGAGGAGTTTCTG*A*AGAT-3′ (forward) and 5′-GTGCCATGATCT*T*CAGAAACTCCTCCT-3′ (reverse).

IMAGE Clone 6177872, containing full length human *EGR2* cDNA, was purchased from MRC geneservice (Cambridge, UK) and subcloned into *EcoR* I/*Not* I-digested pcDNA3.

Human *EGR3* cDNA was obtained by reverse transcription, as described under RT-PCR, of total RNA prepared from Mel888 cells stimulated for 2 hours with PMA using the *EGR3*-specific primer 5′-CCTCAGGCGCAGGTGGTGAC-3′. The obtained *EGR3* cDNA was amplified by PCR using *Pfu* Turbo Polymerase (Stratagene) with the forward primer, including *BamH* I-site, 5′-ATA GGATCC CCCCTGCACCACGGATCGCC-3′ and the reverse primer, including *EcoR* I-site, 5′-ATA GAATTC CCTCAGGCGCAGGTGGTGAC-3′ and subcloned into the *EcoR* I/*BamH* I-digested pcDNA3-vector.

Isolation and characterization of the *NAB2* genomic sequence and the production of *NAB2* 5′ region deletion reporter constructs were described previously (Kumbrink et al., 2005). Inserts of all constructs were validated by sequencing.

Jetstar 2.0 plasmid kit (Genomed, Lohne, Germany) was used for DNA preparation.

In silico analysis

In silico analysis was conducted using the Genomatix suite of programs (Cartharius et al., 2005; Quandt et al., 1995).

Determination of transfection efficiency

Cells were seeded at 3×10^5 cells/well (in 6-well plates) and incubated for 24 hours before transfection with 2 μg of the enhanced green fluorescent protein (EGFP) expression vector pEGFP-N1 (Clontech, USA) using MATra-A reagent (IBA, Goettingen, Germany) according to the manufacturer's recommendations. After 48 hours cells were analyzed by flow cytometry (FACscan, Bectin Dickinson) and Cell Quest software.

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Transfections and Luciferase Assays

Cells were seeded at 1×10^4 cells/well (in 96-well plates) and cultured for 48 hours prior to transfection. For each transfection 2–60 ng of empty and/or expression vector along with 60 or 100 ng of *NAB2* promoter luciferase construct were transfected using MATra-A reagent (IBA, Goettingen, Germany) according to the manufacturer's recommendations. Forty-eight hours post transfection, or as indicated, cells were lysed with cell culture lysis reagent (CCLR, Promega, WI, USA) at RT for 30 min on a shaker. Luciferase activity of all extracts was measured using the firefly luciferase assay system (Promega) and normalized to protein concentration using Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Each experiment was performed in triplicate samples at least twice using different DNA preparations of each construct. In some cases, as indicated cells were seeded at 3×10^5 cells/well (in 6-well plates) and incubated for 24 hours before transfection with 2 μg of the indicated vectors.

siRNA transfections

SK-MEL-23 cells were transfected with 30 nM siRNA using siPORT *NeoFX* (Ambion, Austin, Texas, USA) according to the manufacturer's recommendations in 12-well plates and incubated for 24 hours. SiRNAs directed against *EGR2* (ID # 115177), *EGR3* (ID # 115514), and negative controls (Cat. # 4615 and # 4642) were purchased from Ambion. Sequences of siRNAs specific for *EGR2* and *EGR3*, which are not specific for RNAs of other members of the EGR family were: *EGR2*, 5′-CGCAAAACCACUAACUGAAtt-3′; *EGR3*, 5′-CCAACACAACAGAUAGAAUtt –3′.

RT-PCR

Cells were washed twice with phosphate buffered saline (PBS) and lysed on ice. RNA was extracted using RNAeasy kit from Qiagen (Hilden, Germany). One μg of total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the manufacturer's recommendations. The PCR primers, which were designed to amplify cDNA and not genomic DNA, were: human *EGR2*, 5′-CAACATCTACCCGGTGGAGGAC-3′ (forward) and 5′-GATGCCTGCACTCACAATATTG-3′ (reverse), human *EGR3*, 5′-CCAGCGCGCTCAACCTCTTC-3′ (forward) and 5′-GTAGAGGTCGCCGCAGTTGGAG-3′ (reverse). Primers for *EGR1*, *NAB2*, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were described previously (Kumbrink et al., 2005). To check for linear amplification each PCR reaction was performed with 2 μl and 1 μl of undiluted cDNA and serial dilutions of 1:10, 1:100, and 1:1000. Linear amplification for the majority of the PCR reactions was achieved with 1 μl of 1:10 diluted cDNA. Due to the low expression levels of *EGR2* and *EGR3* in some cell lines these PCR reactions were performed with undiluted cDNA as template. Linear amplification of *GAPDH* with undiluted cDNA (Fig. 2C) was obtained with a 24 cycle PCR reaction. PCR was performed using 1 μl of 1:10 diluted or undiluted cDNA in a 20 μl 30 cycle PCR reaction with the use of *Taq* polymerase and buffer Y from Peqlab (Erlangen, Germany). Products were separated by electrophoresis through a 1 % agarose gel. To control for the integrity and uniformity of the sample preparations, *GAPDH* mRNA was amplified. Densitometric analysis was performed using SigmaGel software (SPSS science).

Real time PCR

Real time PCR was performed utilizing ABI PRISM™ 7000 Sequence Detection System (SDS) Version 1.2.3 (9600 Emulation), TaqMan® Gene Expression Assays, and 2x TaqMan® Universal PCR Master Mix as recommended by the manufacturer Applied Biosystems (Foster City, CA, USA) using 25 ng of cDNA in a 25 μl approach. Comparative Ct method was applied for relative quantitation of each gene expression between samples

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(Livak et al., 2001) and normalized to *glucose-6-phosphate-dehydrogenase* (*G6PD*) levels. TaqMan® Gene Expression Assays used were: *EGR1*, Hs00152928_m1; *EGR2*, Hs00166165_m1; *EGR3*, Hs00231780_m1, *NAB2*, Hs01082003_m1; *G6PD*, Hs00166169_m1. PCR specificity and amplicon size was validated by electrophoresis through a 2 % agarose gel.

RESULTS

Regulation of NAB2 by EGR1, EGR2, and EGR3 in carcinoma and melanoma cells

A previous study showing that EGR1 is an important positive regulator of NAB2 expression in colon carcinoma and melanoma cells pointed to the involvement of additional members of the EGR family in NAB2 regulation (Kumbrink et al., 2005). A comprehensive *in silico* analysis of the *NAB2* 5′ region revealed that the *NAB2* promoter, located from bp −674 to −74 relative to the translation start site (Kumbrink et al., 2005), contains eleven putative EGR1, two EGR2, and two EGR3 sites, most of which overlap with SP1/SP2 sites (Fig. 1). In addition, five sites for cAMP-responsive elements binding proteins (CREB), two ETS family and AP2 sites were identified in the *NAB2* promoter. Of note is a cluster of multiple overlapping binding sites between bp −329 to −260 including five Sp1, five EGR1 motifs, and a single EGR3 and AP2 site, which was shown to be the major regulatory element involved in basal *NAB2* promoter activity as well as EGR1-mediated and phorbol ester induced *NAB2* promoter activation (Kumbrink et al., 2005). Comparative *in silico* analysis of the *NAB2* 5′ region of *Macaca mulatta*, *Bos taurus*, *Rattus norvegicus*, and *Mus musculus* demonstrated a high evolutionary conservation of most of the EGR and SP1 sites including this cluster further pointing to its importance (data not shown).

In order to investigate the role of the EGR family members in the regulation of NAB2 expression the colon carcinoma cell line Colo 320DM, which expresses nearly undetectable levels of the EGR molecules (Fig. 2B), was transfected with expression constructs for human EGR1, EGR2, EGR3, or PHLDA1 (which does not influence *NAB2* promoter activity (Kumbrink et al., 2005)) and empty vector (ev) as controls. The effect on the endogenous *NAB2* expression was examined after 48 h by RT-PCR. As seen in Fig. 2A transfection of either EGR1, EGR2, or EGR3 led to enhanced *NAB2* mRNA expression in comparison to controls. This suggests that, in addtion to EGR1, also EGR2 and EGR3 participate in the positive regulation of *NAB2* transcription.

Although ectopic expression of EGR2 and EGR3 had no effect on *EGR1* levels, transfection of EGR1 led to a slight increase in *EGR2* and a stronger induction of *EGR3* (Fig. 2B). No influence on *EGR3* expression was observed after transfection of EGR2, whereas ectopic expression of EGR3 resulted in enhanced *EGR2* levels. These results suggest that EGR1 is a positive regulator of *EGR2* and *EGR3* transcription and that *EGR2* might be a target gene of EGR3.

Consistent with this are results obtained using a dominant negative (dn) NAB2. dnNAB2, containing an E51K amino acid exchange in the EGR binding domain, integrates into wild type (wt) NAB2 protein complexes thereby inactivating the whole complex and leading to increased EGR activity (Svaren et al., 1998). Since the expression of all three EGR family members is low or undetectable in Colo 320DM cells MCF-7 breast carcinoma cells, which express high *EGR1*, moderate *NAB2*, and low *EGR2*, and *EGR3* levels (Fig. 2C), were transfected with wtNAB2, dnNAB2, or empty vector control. The effects on endogenous *EGR1*, *EGR2*, *EGR3*, and *NAB2* expression were analysed after 48 h by RT-PCR. While overexpression of wild type NAB2 did not significantly effect EGR expression, ectopic expression of dnNAB2 resulted in an increase ($P < 0.05$) in *EGR1*, *EGR2*, and *EGR3* mRNA levels, which was most prominent for *EGR3*. This indicates that endogenous NAB2 proteins

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inhibit not only the activity (Svaren et al., 1998) but also the autoregulated expression of the EGR family members.

To investigate how each of the EGR family members influence *NAB2* promoter activity, human EGR1, EGR2, or EGR3 were transfected together with the human *NAB2* promoter into the colon carcinoma cell lines Colo 320DM, LoVo, and the melanoma cell lines Mel 888 and Mel Ei (Fig. 3A). Transfection efficiencies of the cell lines as determined using a EGFP expression construct were similar (Mel 888, 40%; Mel Ei, 39%; Lovo 30%; Colo 320DM, 45%). Sixty ng of the luciferase reporter construct maluc-679, which contains the *NAB2* promoter, were transfected together with either 40 ng of EGR1, EGR2, EGR3, or PHLDA1 as control. In all cell lines EGR1, EGR2, and EGR3 increased the *NAB2* promoter activity compared to PHLDA1. EGR1- and EGR3-mediated activation was higher than EGR2-dependent activation in each cell line. Transfection of EGR2 resulted in an 1.5 to 5-fold activation of the *NAB2* promoter. Induction of the *NAB2* promoter mediated by EGR1 was 6 to 14-fold in the melanoma cell lines, whereas it was only 2 to 5-fold in the colon carcinoma cells. EGR3-dependent induction was also higher in melanoma cells (7 to 9-fold) than in the colon carcinoma cells (2 to 3-fold). These results indicate not only that EGR1 and EGR3 are more potent activators of *NAB2* transcription than EGR2 but also that EGR1 and EGR3 have a strong influence on the regulation of *NAB2* expression especially in melanoma cell lines. Transfection of Mel 888 cells with increasing amounts (2–40 ng) of either EGR1, EGR2, EGR3, or PHLDA1 as control together with 60 ng of the reporter construct maluc-679 resulted in a dose-dependent activation of the *NAB2* promoter by each of the EGR molecules (Fig. 3B). Although transfection of Mel 888 with 2 ng of EGR1 and EGR2 had no effect on the promoter activity 2 ng of EGR3 were sufficient to activate the *NAB2* promoter by 4-fold ($P < 0.01$), suggesting that EGR3 is the more potent activator of the *NAB2* promoter. These results indicate that EGR1, EGR2, and EGR3 each induce the expression of their coregulator NAB2 by activating the *NAB2* promoter in melanoma and colon carcinoma cells. Similar results were obtained for cervix and breast carcinoma cell lines (data not shown).

EGR1 and EGR3 activate the *NAB2* promoter in concert in melanoma cells

To investigate whether EGR1, EGR2, and EGR3 act simultaneously on the *NAB2* promoter Mel 888 cells were transfected with the *NAB2* promoter together with different combinations of the EGR expression vectors. Fifteen ng of each expression construct were used in these experiments since previous results indicated that transfection of 10 or 20 ng of EGR1, EGR2, or EGR3 expression constructs lead to detectible but not maximal induction of the *NAB2* promoter. Sixty ng of the *NAB2* promoter reporter construct maluc-679 were transfected into Mel 888 cells together with 15 ng of either EGR1, EGR2, EGR3, or PHLDA1 as control (Fig. 3C). Empty vector was added to make a total of 105 ng DNA. Similar to the results in Fig. 3A activation of the *NAB2* promoter was strongest by EGR1 or EGR3 (5-fold), whereas induction by EGR2 was only 3-fold. Concomitant transfection of EGR1 and EGR3 resulted in a doubling of the *NAB2* promoter activity (10-fold) compared to the activation mediated by each transcription factor alone (5-fold). EGR1- and EGR3-dependent promoter induction was not significantly influenced by simultaneous transfection of EGR2. These results suggest that EGR1 and EGR3 act in concert to activate the *NAB2* promoter and exclude mutual inhibition between these factors on the *NAB2* promoter in melanoma cells.

EGR2 and EGR3 are important positive regulators of endogenous *NAB2* expression

The importance of endogenous EGR2 and EGR3 in the regulation of *NAB2* expression was investigated using short-interfering (si) RNAs. The human melanoma cell line SK-MEL-23 was chosen for these experiments as it expresses significant basel levels of NAB2, EGR2

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and EGR3. SK-MEL-23 was transfected with siRNAs directed against either *EGR2* or *EGR3*, or a negative control with no similarity to human gene sequences. After 24 h the effects on *EGR1*, *EGR2*, *EGR3*, and *NAB2* expression were determined by real time PCR (Fig. 3D). Transfection of siRNA directed against *EGR2* resulted in a 46% reduction of *EGR2* expression and a 34% decrease of *NAB2* expression, while *EGR3* expression was reduced only slightly and *EGR1* expression was not influenced. A reduction of *EGR3* expression by 26% by *EGR3*-specific siRNA was associated with a reduction of *NAB2* expression by 38% and *EGR2* levels by 21%, whereas *EGR1* mRNA remained unaffected. Taken together, our results indicate that the transcription factors EGR2 and EGR3 are important for the basal expression of their coregulator NAB2 in melanoma cells.

Identification of EGR2- and EGR3-responsive elements in the *NAB2* promoter

A cluster of multiple EGR/SP1 binding sites and one AP2 site located at bp −329 to −260, in addition to elements located further upstream, was shown to play a crucial role in EGR1-mediated activation of the *NAB2* promoter (Kumbrink et al., 2005). To investigate whether the same or distinct elements in the *NAB2* promoter are necessary for EGR2- and EGR-3-dependent activation Colo 320DM and Mel 888 cells were transfected with 5′ truncated *NAB2* promoter fragments (100 ng) together with 40 ng of expression constructs for EGR2, EGR3, or PHLDA1 as control (Fig. 4). The highest EGR-mediated activity, as normalized to PHLDA1 transfected cells, was obtained in both cell lines with maluc-679, containing the *NAB2* promoter, and this was therefore set as 100%. As seen previously (Fig. 3A) the activation by each factor was higher in Mel 888 cells and EGR3-mediated induction (3 and 4-fold) was higher than EGR2-mediated (1.5 and 2-fold). The EGR2- and EGR3-induced pattern of activity of the 5′ truncated *NAB2* promoter constructs was similar in Mel 888 and Colo 320DM. Serial truncation of the *NAB2* promoter to bp −468 resulted in a successive reduction of EGR-mediated activity. Removal of additional 89 bp to bp −379 had no influence on luciferase activity. This indicates that, as observed for EGR1 (Kumbrink et al., 2005), the EGR1/SP1 binding site at bp −422 is also only of minor importance in response to EGR2 and EGR3, whereas elements further upstream, including two EGR1/SP1 sites, two EGR2 and two CREB sites, an EGR3/SP2 site, an AP2 motif as well as an ETS binding site, contribute significantly to EGR-dependent *NAB2* promoter activation. Further 5′ deletion of the *NAB2* promoter to nucleotide −263 resulted in a strong reduction of luciferase activity, indicating an important element between bp −379 and −263 responsive to EGR2 and EGR3. This region, comprising a cluster of multiple EGR/SP1 binding sites and one AP2 site (bp −329 to −263), was shown previously to be important for EGR1-dependent activation of the *NAB2* promoter (Kumbrink et al., 2005). Although maluc-263 is still responsive to the EGR factors in Colo 320DM, further truncation to bp −195 completely abolished EGR-mediated induction of the *NAB2* promoter, suggesting that two EGR1/SP1 sites as well as one ETS and CREB motif, located from bp −195 to −74, have no function in EGR-mediated *NAB2* promoter activation in Colo 320DM cells. In contrast to this, in Mel 888 cells a significant activation of maluc-195 was observed in response to EGR2 (2.5-fold) and EGR3 (4-fold). Further addition of 68 bp to bp −263 resulted in the strongest EGR2-mediated activation (5-fold) observed in the *NAB2* promoter deletion constructs leading to 30% of the maximum activity induced by EGR2. This indicates the importance of two overlapping EGR1/SP1 sites and one CREB motif between bp −263 and −195 in response to EGR2 in Mel 888 cells. Although in Colo 320DM the complete promoter up to bp −679 is required for maximum EGR2-induced activity in Mel 888 maluc-558 was sufficient, thus indicating the significance of an EGR3/SP2 and an EGR1/SP1 site between bp −558 and −468 in response to EGR2 in Mel 888 cells. However, these cis-regulatory elements mediated the highest EGR3-induced activity (4.5-fold) in Colo 320DM, showing that in Colo 320DM cells this region is preferentially responsive to EGR3 whereas it is important for both factors in Mel 888. While in Mel 888 cells the EGR3-mediated fold activation of maluc-558 was similar to

that obtained with maluc-679 the induction of maluc-679 by EGR3 is reduced in Colo 320DM cells by 35%. Since elements located from bp −679 to −558 are necessary in both cell lines for the maximum *NAB2* promoter activity in response to EGR3, this suggests the involvement of other factors recruited to this region, which attenuate the EGR3-mediated induction of the *NAB2* promoter in Colo 320DM cells. The region between bp −679 and −558 contains two EGR2 and two CREB sites, an EGR1/SP1 site, an AP2 motif, as well as an ETS binding site.

In summary, the pattern of activation of the *NAB2* promoter deletion constructs by EGR2 and EGR3 was similar to that previously described for phorbol esters and EGR1 (Kumbrink et al., 2005). The major regulatory element at bp −329 to −263, comprising a cluster of multiple Egr/Sp1 binding sites and one AP2 site, was shown to be important for EGR1, EGR2, as well as EGR3-induced activity. Furthermore, the region between bp −679 and −558, including two EGR2 and two CREB sites, an EGR1/SP1 site, an AP2 motif as well as an ETS binding site, might contribute to the stronger activation of the *NAB2* promoter by EGR3 detected in melanoma cell lines.

The role of the EGR transcription factors in NAB2 induction in response to phorbol esters

The expression kinetics of the EGR transcription factors and NAB2 are highly variable depending on cell type and stimulus (Beckmann et al., 1997; Collins et al., 2008; Honkaniemi et al., 2000). In order to gain insight into the temporal regulation of *NAB2* transcription a comprehensive analysis of the expression kinetics of *EGR1*, *EGR2*, *EGR3*, and *NAB2* in response to the phorbol ester PMA was performed. Expression levels were monitored over 12 time points in three melanoma and two colon carcinoma cell lines using RT-PCR (Fig. 5). In Mel 888 cells a transient induction of *EGR1*, *EGR2*, *EGR3*, and *NAB2* was observed. *EGR1* levels were increased after 15 min reaching its maximum after 30 min. *EGR2* and *EGR3* expression were induced after 30 min. While *EGR2* levels peaked after 45 min, the maximum expression of *EGR3* was detected after 1.5 h. Due to the high basal levels only a weak induction of *NAB2* was observed after 1 h reaching its maximum after 2 h. After 6 to 10 h the levels of *EGR1*, *EGR2*, *EGR3*, and *NAB2* declined. A similar kinetic was observed for a second melanoma cell line Mel Ei (data not shown). In comparison to Mel 888 a delayed induction of *EGR1*, *EGR2*, and *EGR3* expression, which was associated with a postponed *NAB2* induction, was observed in the colon carcinoma cell lines Colo 320DM and LoVo (data not shown). In Mel JuSo the maximum expression of *EGR3* as well as that of *NAB2* in response to PMA ocurred 45 min earlier compared to Mel 888, emphasizing the importance of *EGR3* in the induction of *NAB2*. In contrast to the transient PMA-mediated activation of *EGR1*, *EGR2*, *EGR3*, and *NAB2* expression in Mel 888, the induced mRNA expression of all factors remained stable for 14 hours in Mel JuSo. In Colo 320DM a prolonged induction of *EGR1* but not of *NAB2* was observed, suggesting that EGR2 and EGR3 are necessary to maintain NAB2 expression. These results suggest that EGR1 seems to be important in a rapid but low NAB2 induction, whereas EGR2 and EGR3 are apparently necessary for the maximum induction and maintenance of NAB2 expression in response to phorbol esters.

EGR1-, EGR2-, and EGR3-mediated induction of the *NAB2* promoter is inhibited by Nab corepressors

Nab1 has been shown to act as a repressor of Egr1 and Egr2 but not of Egr3 (Russo et al., 1995), whereas NAB2 is able to repress Egr3 as well (Sevetson et al., 2000; Svaren et al., 1996; Svaren et al., 1998). To determine the influence of NAB1 and NAB2 on EGR1-, EGR2-, and EGR3-induced *NAB2* promoter activity in melanoma cells, 60 ng of the reporter construct maluc-679 was cotransfected with either 40 ng EGR1, EGR2, EGR3, or PHLDA1 as control and 40 ng NAB1 and/or NAB2 into Mel 888 cells (Fig. 6A). Emtpy vector was

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used to make a total of 180 ng of DNA. Expression of NAB1 reduced the 9-fold EGR1-mediated induction by 75% and the 2-fold EGR2-mediated activation by 54%, whereas the EGR3-mediated induction (8-fold) was not significantly affected. In contrast, transfection of NAB2 completely inhibited the activation of the *NAB2* promoter by each of the EGR molecules. EGR2-mediated activation was reduced by NAB2 slightly below the basal *NAB2* promoter activity. No further reduction of *NAB2* promoter activity was observed after cotransfection of NAB1 and NAB2. Additional transfection of increasing amounts (2–60 ng) of NAB2 together with 40 ng EGR1, EGR3, or PHLDA1 as control resulted in a dose-dependent inhibition of EGR1- and EGR3-mediated *NAB2* promoter activity, which was complete at 40 ng (Fig. 6B). These results indicate that NAB2 can completely repress EGR1-, EGR2-, and EGR3-mediated activation of the *NAB2* promoter, whereas NAB1 is a less potent inhibitor of only EGR1 and EGR2.

DISCUSSION

The EGR family of transcription factors, comprising EGR1 (NGFI-A, Krox24), EGR2 (Krox20), and EGR3 (PILOT), play key roles in the regulation of proliferation, differentiation, and other cellular responses to extracellular stimuli (Carter et al., 2007; Thiel et al., 2002; Unoki et al., 2003). The activity of the EGR family members is controlled in part through the binding of NAB2 (Russo et al., 1995; Sevetson et al., 2000; Svaren et al., 1996; Svaren et al., 1998).

Recent studies have shown that EGR1 itself induces the expression of NAB2 by binding to the human *NAB2* promoter following stimulation with phorbol esters in melanoma and colon carcinoma cells (Kumbrink et al., 2005).

How EGR2 and EGR3 influence Nab2 expression appears to depend on cell type specific programs. Enhanced *Nab2* mRNA was observed in human breast cancer cells transfected with EGR3 (Inoue et al., 2004) and in rat Schwann cells transfected with Egr2 (Nagarajan et al., 2001). However, depletion of Egr2 did not influence Nab2 levels in myeloid progenitors (Laslo et al., 2006) and in CD4 T lymphocytes Egr2 and Egr3 were shown to inhibit Nab2 expression (Collins et al., 2008). This suggests a distinct regulation of the NAB2/EGR system in hematopoietic cells and cells of neuroectodermal and epithelial origin.

To address this question we have analyzed the regulation of NAB2 expression by members of the EGR transcription factor family in cells of neuroectodermal and epithelial origin. Transient transfections showed that EGR1, EGR2, and EGR3 activate the *NAB2* promoter in these cells (Fig. 3). EGR1 and EGR3 are more potent activators than EGR2. Cis-regulatory elements in the *NAB2* promoter described to be responsive to EGR1 and phorbol esters (Kumbrink et al., 2005), including the evolutionary conserved major regulatory element at bp −329 to −263, were found to also be important for EGR2- and EGR3-induced activity (Fig. 4). This major regulatory element is comprised of a cluster of multiple overlapping EGR/SP1 binding sites. EGR2 was shown to be recruited to this element in the rat *Nab2* promoter after treatment with neuregulin (Srinivasan et al., 2007). The importance of the three EGR family members as activators of endogenous NAB2 expression was shown using transfection of specific siRNAs (Kumbrink et al., 2005) (Fig. 3D). Depletion of EGR2 or EGR3 in SK-MEL-23 melanoma cells reduced not only *EGR2* or *EGR3* levels but also *NAB2* levels.

Taken together these results indicate that in melanoma and carcinoma cells EGR1, EGR2, and EGR3 each activate the *NAB2* promoter and lead to mRNA expression. In turn the *NAB2* promoter activity induced by EGR1, EGR2, and EGR3 in these cells is inhibited by NAB2 (Fig. 6A).

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In addition to the reduction in *NAB2* expression, transfection of EGR3 siRNA also led to a decrease in *EGR2* levels, whereas *EGR1* levels remained unaffected, indicating that *EGR2* is a target gene of EGR3. Consistent with this, ectopic expression of EGR3 resulted in an increase of endogenous *EGR2* levels (Fig. 2B) as has also been observed in T cells (Collins et al., 2008). However, in contrast to T cells where overexpression of Egr3 leads to a downregulation of Nab2 and Egr1, in carcinoma and melanoma cells EGR3 activated *NAB2* mRNA expression and had no influence on *EGR1* expression. After transfection of EGR1 the endogenous *EGR3* and *EGR2* expression in colon carcinoma (Fig. 2B) and Egr3 expression in neuroblastoma cells were increased (Ehrengruber et al., 2000). Derepression of EGR-activity by dominant negative NAB2 led to enhanced endogenous *EGR1*, *EGR2*, and *EGR3* levels in breast carcinoma cells (Fig. 2C). The increase in *EGR2* and *EGR3* expression can be explained by the enhanced activity of the highly expressed EGR1 (which induces *EGR2* and *EGR3*) and EGR3 which induces *EGR2* (Fig. 2B and summarized in Fig. 7). Neither transcription factor overexpression studies nor siRNA silencing experiments indicated that EGR2 or EGR3 influence *EGR1* expression. However, EGR1 has been shown to be recruited to its own promoter in response to growth factors (Langmann et al., 2009;Sakamoto et al., 1994) suggesting that EGR1 can induce its own expression. These observations point to a positive autoregulation within the EGR family which might result in a stronger and maintained NAB2 induction in response to environmental stimuli or a constitutive NAB2 expression as observed in malignant melanomas (Kirsch et al., 1996).

The transient induction of EGR1 in response to environmental stimuli usually occurs after minutes and is followed one to two hours later by an increase of NAB2 expression (Kumbrink et al., 2005; Thiel et al., 2002). However, the expression kinetics and inducibility of the EGR family members are highly variable depending on cell type and stimulus (Collins et al., 2008; Honkaniemi et al., 2000; von der Kammer H. et al., 1998). To date the most extensive study on the expression pattern and functional interactions between the Egr family members and Nab2 has been conducted in CD4 T lymphocytes (Collins et al., 2008). In these cells the maximum *Egr1* and *Egr3* induction in response to the phorbol ester PMA occurs early and concurrently, while *Egr2* and *Nab2* expression peaks 2 hours later. In contrast, our studies demonstrate a different kinetic picture in cells of neuroectodermal and epithelial origin (Fig. 5). PMA treatment resulted in an early induction of *EGR1* followed by an increase in *EGR2*, *EGR3*, and finally *NAB2* levels. High and prolonged *NAB2* levels were associated with strong *EGR2* and *EGR3* expression. This suggests that unlike in T cells, where Egr2 and Egr3 inhibit Nab2 expression, in neuroectodermal and epithelial cells EGR2 and EGR3 are necessary for the maximum induction and maintenance of NAB2 levels.

Taken together our data show that EGR1, EGR2, and EGR3 are important cooperative positive regulators of basal as well as inducible *NAB2* transcription in melanoma and carcinoma cells. Nevertheless, other transcription factors certainly also play a role. In addition to the large number of EGR/SP1 binding sites, motifs for CREB, ETS family members, NFκB, and AP2 were also identified in the *NAB2* promoter or in close vicinity. Recent studies demonstrated a coregulation of *NAB2* transcription by two ETS factors, Ets2 and Etv1, and Egr2 in rat Schwann cells (Srinivasan et al., 2007). The inducible ETS proteins and Egr2 seem to be able to act independently but also synergistically on the *Nab2* promoter. Although, in contrast to several ETS sites present in the rat *Nab2* promoter (Srinivasan et al., 2007), only two putative binding sites for ETS factors were identified in the human *NAB2* promoter, these studies suggest the ETS factors may also be important regulators of *NAB2* transcription in humans.

Several studies have demonstrated, in contrast to the major definition of NAB2 as a corepressor of the EGR transcription factors (Kumbrink et al., 2005; Lucerna et al., 2003; Silverman et al., 1999; Svaren et al., 1996), that NAB2 can act also as a coactivator of EGR-

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mediated promoter activation (Collins et al., 2006; Sevetson et al., 2000). However, the studies presented here and previous studies with EGR1 (Kumbrink et al., 2005) showed that EGR1, EGR2, and EGR3-mediated induction of the *NAB2* promoter was completely inhibited by NAB2 in melanoma cells (Fig. 6).

In conclusion, our results (as depicted in Fig. 7) indicate that in cells of neuroectodermal and epithelial origin, EGR1, EGR2, and EGR3 autoregulate each other and induce the expression of *NAB2* which results in the establishment of a negative feedback loop to limit the activity on the *NAB2* promoter and the expression of the EGR family members in response to environmental stimuli. This is further supported by a recent study showing that the Egr transcription factors are required for epidermal growth factor (EGF)-mediated proliferation of astrocytes which is blockable by Nab2 (Mayer et al., 2009). These findings and the results presented here point to a distinct EGR/NAB2 function in neuroectodermal and epithelial cells as compared to hematopoetic cells.

Since a dysregulation of the NAB2/EGR system is associated with several malignancies our studies might open new avenues to develop therapeutic strategies. In malignant melanoma NAB2 is overexpressed (Kirsch et al., 1996) whereas in the majority of prostate carcinomas NAB2 expression is lost (Abdulkadir et al., 2001a) and enhanced EGR1 activity promotes tumorigenesis (Abdulkadir et al., 2001b) and growth under androgen-depleted conditions (Yang et al., 2006). EGR3 was shown to play an important role in estrogen-meditated invasion (Inoue et al., 2004) and was recently identified as an independent prognostic factor in breast carcinoma (Suzuki et al., 2007). NAB2 (Lucerna et al., 2003), EGR1 (Lee et al., 2009), and EGR3 (Liu et al., 2008) are all involved in the regulation of VEGF-mediated angiogenesis and thus targeted modulation of the NAB2/EGR system could be useful in influencing angiogenic activity not only in tumors but also in cardiovascular disease. Furthermore, Egr2-Nab interaction has been shown to be critical for proper peripheral nerve myelination (Baloh et al., 2009; Desmazieres et al., 2008). Thus, understanding the detailed mechanism of the cell type and disease specific NAB2/EGR relationships can have important clinical implications.

Acknowledgments

Contract grant sponsor: Wilhelm Sander Stiftung; Contract grant number: 1997.044.02.

Contract grant sponsor: Friedrich Baur Stiftung; Contract grant number: 0013/2008.

Contract grant sponsor: NIH/NCI; Contract grant number: CA106468.

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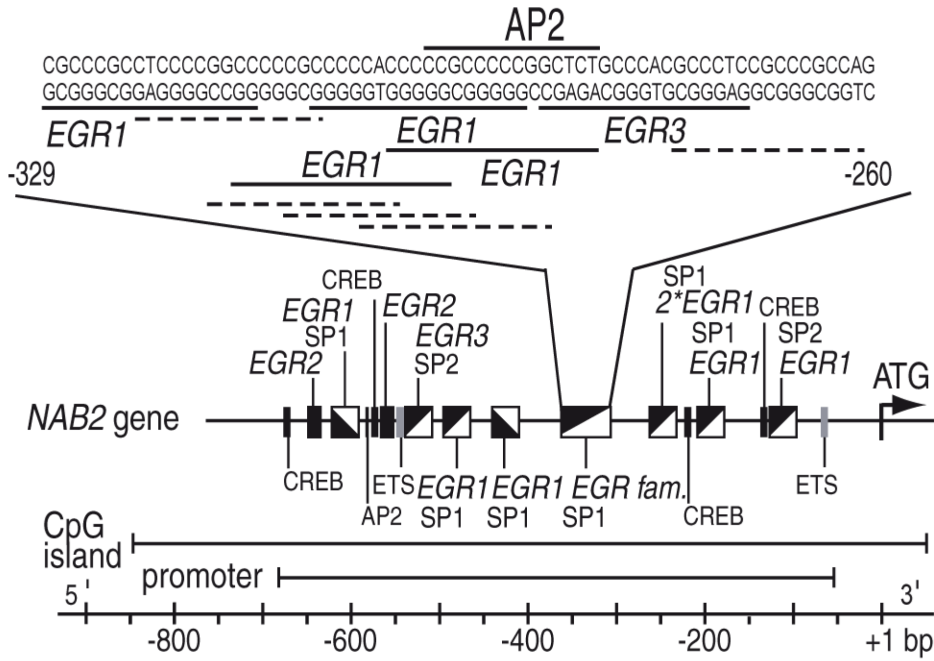

Fig. 1.
Schematic representation of the *NAB2* 5′ region. Numbering is in relation to translation start site (+1). Identified promoter region, CpG island, and putative transcription factor binding sites are indicated. EGR family member sites are highlighted in italics. The detailed sequence of the major regulatory element in the *NAB2* promoter between bp −329 and −260 including putative binding motifs (dotted line = SP1 binding site, solid line = EGR family member site) is displayed.

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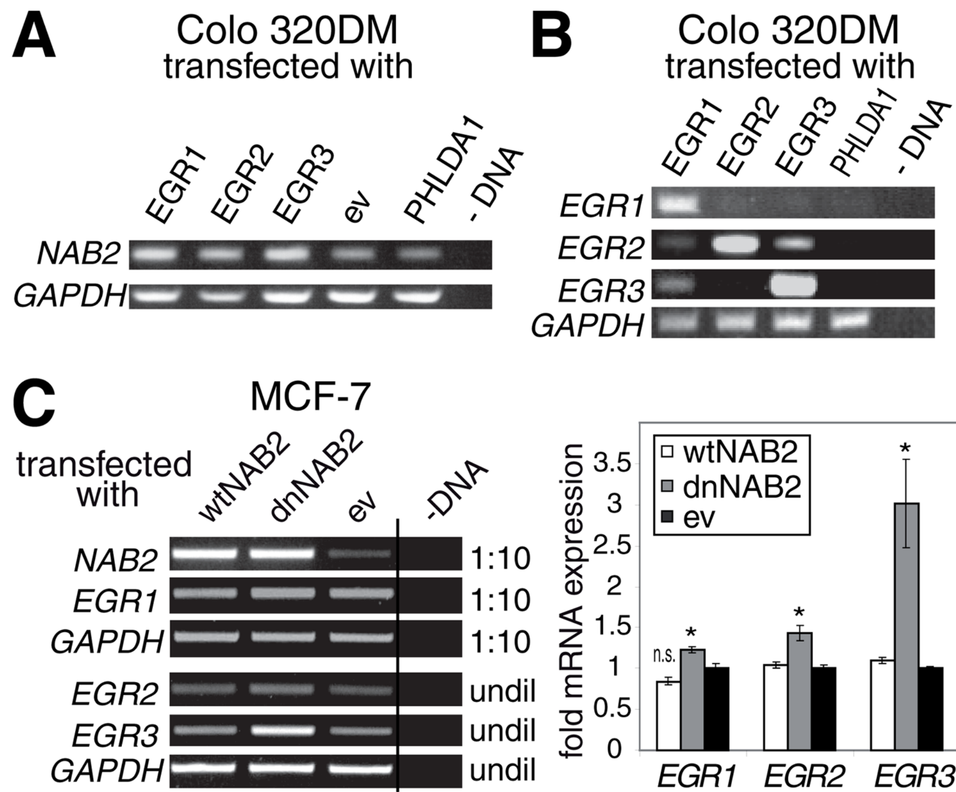

Fig. 2.

A. EGR1, EGR2, and EGR3 induce endogenous *NAB2* expression. Colo 320DM cells were transfected in 6-well plates with 2 μg of expression constructs for human EGR1, EGR2, EGR3, or PHLDA1 and empty vector (ev) as controls. Levels of *NAB2,* and *GAPDH* mRNA were determined by RT-PCR after 48 h as described in "Experimental Procedures". –DNA, control for PCR. *B.* Autoregulation of EGR family members. Colo 320DM cells were transfected in 6-well plates with 2 μg of expression constructs for human EGR1, EGR2, EGR3, or PHLDA1 as control. Levels of *EGR1*, *EGR2*, *EGR3*, and *GAPDH* mRNA were determined by RT-PCR after 48 h as described in "Experimental Procedures". –DNA, control for PCR. *C.* Inhibition of endogenous NAB2 activity enhances *EGR1*, *EGR2*, and *EGR3* expression. MCF-7 cells were transfected in 6-well plates with 2 μg of expression constructs for human wild type wtNAB2, dominant negative dnNAB2, or empty vector (ev) as control. Left panel, levels of *EGR1*, *EGR2*, *EGR3*, *NAB2,* and *GAPDH* mRNA were determined by RT-PCR after 48 h as described in "Experimental Procedures". –DNA, control for PCR. Undiluted (undil) or 1:10 diluted cDNA was used as PCR template as indicated. Right panel, densitometric analysis of *EGR1*, *EGR2*, and *EGR3* mRNA expression after transfection of the indicated construct (as described in "Experimental Procedures"). Results are presented as fold expression in relation to cells transfected with empty vector (ev) control. * $P < 0.05$, n.s., not significant (ev control versus wtNAB2 or dnNAB2). P values were calculated using Student's t-test. A, B, and C. Results shown are from one representative experiment out of three performed.

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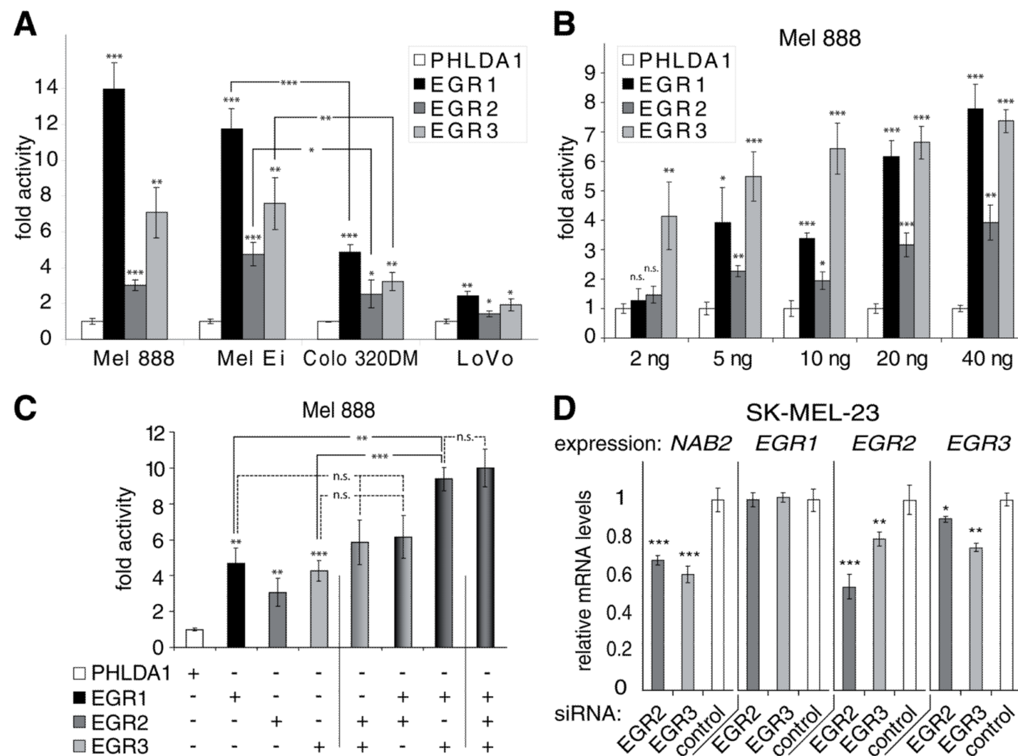

Fig. 3.

EGR1, EGR2, and EGR3 are important regulators of *NAB2* transcription. *A*. EGR1, EGR2, and EGR3 activate the *NAB2* promoter. Mel 888, Mel Ei, Colo 320DM, and Lovo cells were transfected with 60 ng of maluc-679, which contains the human *NAB2* promoter, and 40 ng of either human EGR1, EGR2, EGR3, or PHLDA1 control DNA. Luciferase activity was determined as described in "Experimental Procedures". Results are presented as fold activity over luciferase activity of maluc-679 cotransfected with PHLDA1. Average and standard deviation of triplicates from a representative experiment is shown. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ of PHLDA1 versus EGR1, EGR2, EGR3 or as indicated by connecting lines. *P* values were calculated using Student's *t*-test. *B*. EGR1, EGR2, and EGR3 activate the *NAB2* promoter in a dose-dependent manner. Mel 888 cells were transfected with 60 ng of maluc-679, which contains the human *NAB2* promoter, and increasing amounts, as indicated, of either human EGR1, EGR2, EGR3, or PHLDA1 control DNA. Empty vector DNA added to make a total of 100 ng. Luciferase activity was determined as described in "Experimental Procedures". Results are presented as fold activity over luciferase activity of maluc-679 cotransfected with PHLDA1. Average and standard deviation of triplicates from a representative experiment is shown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, n.s., not significant (PHLDA1 versus EGR1, EGR2, or EGR3). *P* values were calculated using Student's *t*-test. *C*. EGR1 and EGR3 act in concert to activate the *NAB2* promoter. Mel 888 cells were transfected with 60 ng of maluc-679 and different combinations, as indicated, of 15 ng of human EGR1, EGR2, EGR3, or PHLDA1 control plasmid DNA. Vector DNA was added to make a total of 105 ng. Luciferase activity was determined as described in "Experimental Procedures". Results are presented as fold activity over luciferase activity of maluc-679 cotransfected with PHLDA1 control plasmid DNA. Average and standard deviation of triplicates from a representative experiment is shown. ** $P<0.01$, *** $P<0.001$, n.s., not significant of PHLDA1 versus EGR1, EGR2, EGR3 or as indicated by connecting lines. *P* values were calculated using Student's *t*-test. *D*. Depletion of *EGR2* or *EGR3* reduces *NAB2* expression in the human melanoma cell line SK-MEL-23. SK-MEL-23

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cells were transfected with negative control siRNA or siRNAs directed against *EGR2* or *EGR3* as described in “Experimental Procedures”. 24 hours later mRNA expression of *NAB2*, *EGR1*, *EGR2*, and *EGR3* was determined using real time PCR (as described in “Experimental Procedures”). Average mRNA expression in relation to negative control (black bar) and standard deviation of triplicates from one representative experiment out of two performed is shown. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ of control siRNA versus siRNA against EGR2 or EGR3. *P* values were calculated using Student’s *t*-test.

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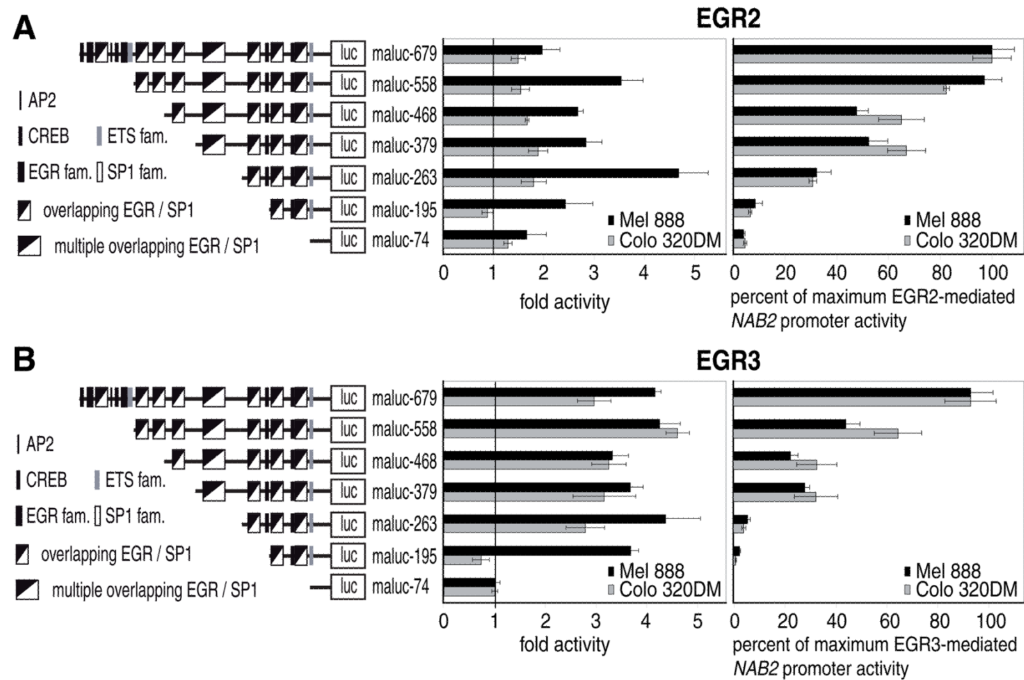

Fig. 4.

Identification of EGR2- (*A.*) and EGR3- (*B.*) responsive elements in the *NAB2* promoter. Mel 888 and Colo 320DM cells were transfected with 100 ng of the indicated promoter reporter construct and 40 ng of human EGR2, EGR3, or PHLDA1 as control as described in "Experimental Procedures". The results are presented as the mean and standard deviation of triplicates from a representative experiment. Left panel, results are presented as EGR2- or EGR3-mediated fold activity compared to PHLDA1 control plasmid (set as 1) of the indicated reporter constructs. Right panel, results are shown as percent of the maximum EGR2- or EGR3-mediated *NAB2* promoter activty obtained with maluc-679, which was set as 100%. Before calculating the percentages the luciferase activity of the PHLDA1-transfected cells was subtracted from the luciferase activity of the *NAB2* promoter constructs.

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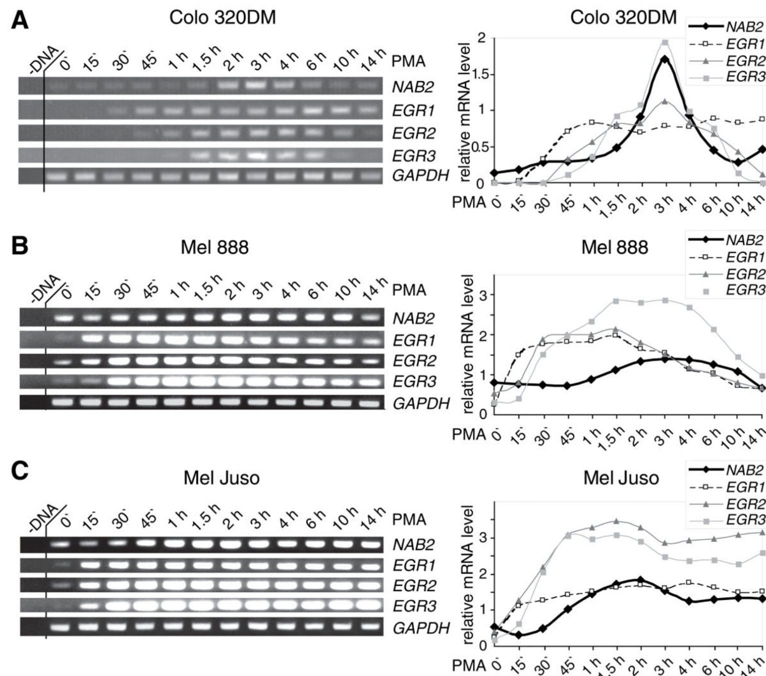

Fig. 5.

Expression kinetics of *EGR1*, *EGR2*, *EGR3*, and *NAB2* in melanoma and colon carcinoma cells in response to phorbol esters. Colo 320DM (*A*.), Mel 888 (*B*.), and Mel JuSo (*C*.) cells were either stimulated for 15 min to 14 h with PMA (as indicated) or left untreated (0′) as described in "Experimental Procedures". Results are presented from one representative experiment out of at least two performed. Left panel, expression of *EGR1*, *EGR2*, *EGR3*, *NAB2*, and *GAPDH* mRNA in the indicated cell lines was determined by RT-PCR (as described in "Experimental Procedures"). –DNA, control for PCR. Right panel, densitometric analysis of *EGR1*, *EGR2*, *EGR3*, and *NAB2* mRNA expression as indicated (as described in "Experimental Procedures"). Results are presented as the ratio of *EGR1*, *EGR2*, *EGR3*, or *NAB2* to *GAPDH* levels.

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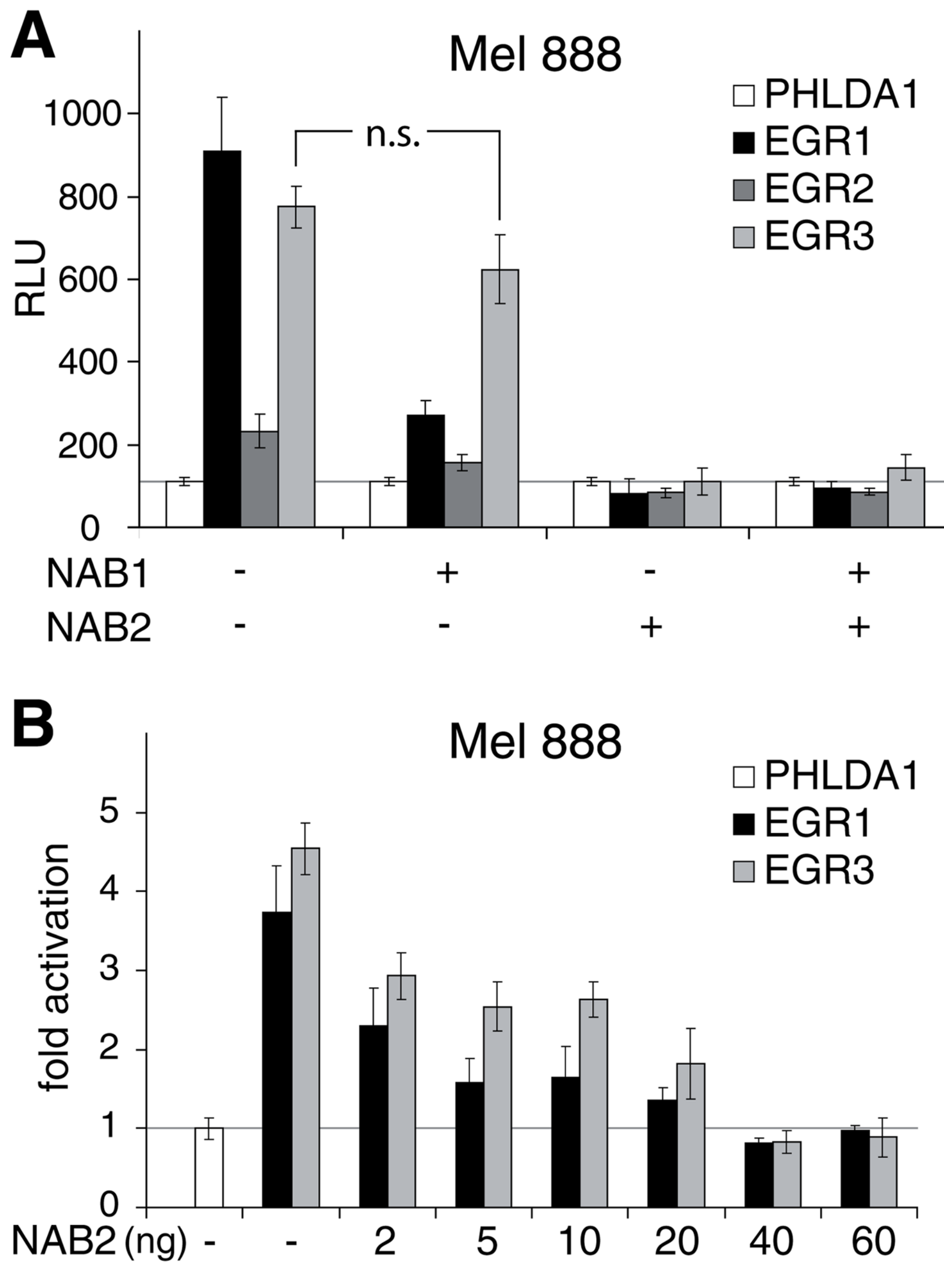

Fig. 6.

EGR1-, EGR2-, and EGR3-mediated induction of the *NAB2* promoter is inhibited by NAB corepressors. *A*. Mel 888 cells were transfected with 60 ng of maluc-679 and 40 ng of expression constructs for human EGR1, EGR2, EGR3, NAB1, NAB2, or PHLDA1, as control for basal promoter activity, as indicated. Vector DNA was added to make a total of 180 ng. Luciferase activity was determined as described in "Experimental Procedures". The results are presented as the mean and standard deviation of triplicates from a representative experiment. RLU, relative light units/μg protein. n.s., not significant (P >0.05). P values were calculated using Student's t-test. *B*. Mel 888 cells were transfected with 60 ng of maluc-679 and 40 ng EGR1, EGR3, or PHLDA1, as control, and increasing amounts of human NAB2, as indicated in ng DNA. Vector DNA was added to make a total of 160 ng. Luciferase activity was determined as described in "Experimental Procedures". Results are

presented as fold activity over luciferase activity of maluc-679 cotransfected with PHLDA1. Average and standard deviation of triplicates from a representative experiment is shown.

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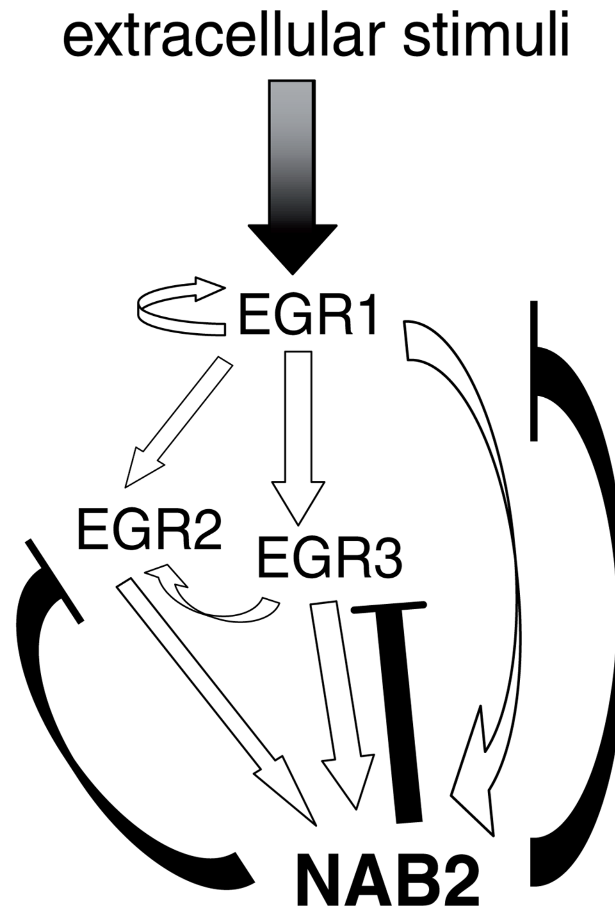

Fig. 7.

Model of the EGR/NAB2 negative feedback system in cells of neuroectodermal and epithelial origin. Arrows represent activation of transcription and barred lines indicate repression of transcriptional activity and expression.