MAML1 Acts Cooperatively with EGR1 to Activate EGR1-Regulated Promoters: Implications for Nephrogenesis and the Development of Renal Cancer

Magnus L. Hansson, Stefanie Behmer, Rebecca Ceder, Sadollah Mohammadi, Giulio Preta, Roland C. Grafström, Bengt Fadeel, Annika E. Wallberg*

Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

Abstract

Mastermind-like 1 (MAML1) is a transcriptional coregulator of activators in various signaling pathways, such as Notch, p53, myocyte enhancer factor 2C (MEF2C) and beta-catenin. In earlier studies, we demonstrated that MAML1 enhanced p300 acetyltransferase activity, which increased the acetylation of Notch by p300. In this study, we show that MAML1 strongly induced acetylation of the transcription factor early growth response-1 (EGR1) by p300, and increased EGR1 protein expression in embryonic kidney cells. EGR1 mRNA transcripts were also upregulated in the presence of MAML1. We show that MAML1 physically interacted with, and acted cooperatively with EGR1 to increase transcriptional activity of the EGR1 and p300 promoters, which both contain EGR1 binding sites. Bioinformatics assessment revealed a correlation between p300, EGR1 and MAML1 copy number and miRNA alterations in renal clear cell carcinoma and p300, EGR1 and MAML1 gene alterations were associated with increased overall survival. Our findings suggest MAML1 may be a component of the transcriptional networks which regulate EGR1 target genes during nephrogenesis and could also have implications for the development of renal cell carcinoma.

Introduction

Mastermind-like 1 (MAML1) is the human homolog of Drosophila Mastermind, a neurogenic gene genetically linked to Notch function [1,2]. The Notch signaling pathway plays an important role in many developmental processes by influencing cellular proliferation, differentiation and apoptosis. In the nucleus, the Notch intracellular domain (Notch ICD) associates with the transcription factor CSL (also known as RBP-Jk or CBF1 in vertebrates, Suppressor of Hairless in Drosophila and Lag-1 in C. elegans) when it is bound to DNA, and coactivators such as PCAF [3], GGN5 [3], p300 [4] and MAML1 [1,2] are recruited to activate the expression of Notch target genes. Recently, MAML1 has been shown to function as a coactivator for transcription factors involved in a variety of Notch-independent signaling pathways, including myocyte enhancer factor 2C (MEF2C) [5], p53 [6] and beta-catenin [7], and the N-terminus of MAML1 is crucial for these interactions. These findings suggest that MAML1 functions as a coactivator in diverse cellular processes and may be a mediator of crosstalk between different signaling pathways.

MAML1 may also affect various signaling pathways by interacting with p300, a commonly used coactivator. We and other researchers have previously reported that MAML1 mediates Notch ICD-mediated transcription in vivo, and can also form chromatin templates in vitro, by recruiting p300 to a DNA-CSL-Notch complex [8,9,10]. We recently reported that MAML1 enhances p300 autoacetylation, histone acetyltransferase (HAT) activity and coactivator function [11]. We also found that p300 acetylates the Notch1 ICD in cell culture assays and in vitro, and that regions located within the Notch C-terminus are essential for Notch acetylation. MAML1 and CSL, components of the Notch transcription complex, strongly enhance Notch acetylation and we suggested that MAML1 increases Notch acetylation by potentiating p300 autoacetylation [12]. p300 can also acylate MAML1, although the effect of this posttranslational modification on the function of MAML1 is currently unknown. MAML1 is a phosphoprotein, and we previously identified GSK3ß as one kinase responsible for phosphorylation of MAML1 in vitro [13]. The active form of GSK3ß directly interacts with the N-terminus of MAML1 to inhibit MAML1 transcriptional activity [13]. In addition, the MAML1 N-terminus is subject to SUMOylation, which also represses the transcriptional activity of MAML1 [14].

The transcription factor early growth response-1 (EGR1) is expressed in response to various extracellular signals, such as growth factors, cytokines, irradiation, and various kinds of stress. In many normal cells, growth factor stimulation rapidly induces expression of EGR1, which subsequently leads to the activation of downstream growth pathways [15]. However, EGR1 can also suppress growth when overexpressed in transformed cells in several experimental systems [16]. The cellular response to EGR1 with respect to apoptosis varies: EGR1 can induce apoptosis by stimulating either p53 or PTEN; however, EGR1 can also

Citation: Hansson ML, Behmer S, Ceder R, Mohammadi S, Preta G, et al. (2012) MAML1 Acts Cooperatively with EGR1 to Activate EGR1-Regulated Promoters: Implications for Nephrogenesis and the Development of Renal Cancer. PLoS ONE 7(9): e46001. doi:10.1371/journal.pone.0046001

Editor: Alejandro H. Corvalan, Pontificia Universidad Catolica de Chile, Chile

Received April 23, 2012; Accepted August 27, 2012; Published September 27, 2012

Copyright: © 2012 Hansson et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors received support from the Swedish Research Council, Swedish Cancer Society and the Swedish Children’s Cancer Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Annika.Wallberg@ki.se
promote survival in certain cell types by counteracting p53-dependent apoptosis [17]. EGR1 plays an important role as a tumor suppressor in breast, brain and lung cancer, as EGR1 is poorly expressed in these tissues and acts as a suppressor of growth and transformation when overexpressed [16]. In contrast, expression of EGR1 appears to be maintained in a high proportion of prostate and kidney cancers, where it promotes growth. EGR1 is absent or expressed at low levels in normal prostate tissues; whereas the EGR1 promoter is regulated by a positive feedback loop between EGR1 and growth factors in prostate cancer cells, which results in constitutive growth. Prostate cancer tissues also express high levels of p300, which leads to constitutively high levels of stable acetylated EGR1 protein, which is an important component in the transformation and progression of this disease [18]. The levels of EGR1 increase with the degree of malignancy in prostate cancer, as indicated by the Gleason tumor score [15]. The ability to reduce EGR1 expression may provide an efficient clinical treatment for prostate cancer, as downregulation of EGR1 may reduce growth factor induction and positive feedback to the EGF1 promoter.

EGR1 has been implicated as an important factor in nephrogenesis and the development of renal cancer. During embryogenesis, EGR1 is expressed in almost all proliferating cells including metanephrine blasts; however, it is normally downregulated during renal development [19]. Expression of EGR1 is also elevated in many Wilms’ tumors compared to normal kidney tissues. Wilms’ tumor is a pediatric malignancy of the kidney, which is often of embryonic origin [20]. Overexpression of EGR1 has been reported to increase proliferation, enhance tumor growth and antagonize the effects of the tumor suppressor Wilms tumor 1 (WT1) in baby rat kidney cells [21]. Renal cell carcinoma (RCC) is the most common kidney cancer in adults, accounting for 80–85% of primary kidney malignancies. Clear cell (conventional) RCC and renal papillary cell carcinoma are the most and second-most frequent types of RCC, respectively. Strefford and colleagues reported that chromosome 5 was overrepresented and rearranged in a significant proportion of 19 renal cell carcinoma cell lines, with the genes encoding EGR1 (5q1.1) and CSF1R (Colonystimulating factor 1 receptor) (5q33-q35) most frequently altered on chromosome 5, further supporting a role for EGR1 in renal cancer [22]. In a previous study, we reported that MAML1 increased p300 acetylation and p300 acetylation activity in embryonic kidney (HEK293) cells [11]. The objective of the present study was to investigate whether MAML1 and p300 regulate the levels of EGR1 in kidney cells.

Materials and Methods

Plasmids

pcDNA3.1-FLAG-EGR1 and pGL3-p300 were purchased from Addgene (Cambridge, MA, USA). The plasmid 4xEBS1-luc was a kind gift from Dr. G. Thiel (University of Saarland Medical Center, Homburg, Germany) and pGL3-EGR1 was generously provided by Dr. T.E. Eling (National Institute of Environmental Health Sciences, NC, USA). pcDNA3.1-FLAG-MAML1 (1–1016), (1–625) and cmv-p300-HA have been described previously [11]. The cDNA encoding MAML1 (1–127) and (75–1016) were amplified by PCR and subcloned into the expression vector FLAG-pcDNA3.1.

Cell lines

The MAML1 cell lines were constructed by transfecting human embryonic kidney (HEK)-293 cells with the vectors pcDNA3.1-FLAG-MAML1 or pcDNA3.1-MAML1 and stable clones were selected with genetin [11].

siRNA transfection

HEK-293 cells were transiently transfected with 100 nM MAML1 siRNA (predesigned MAML1 SMARTpool set of 4 siRNAs; Dharmacon) or control siRNA using DharmaFECT 1 siRNA reagents (Dharmacon, Lafayette Colorado, USA), and cultured in the presence or absence of 50 ng/ml TPA (12-O-Tetradecanoylphorbol-13-Acetate) (Cell Signaling Technology, Boston, MA, USA).

Reporter gene assay

HEK-293 cells in 24-well plates were transiently transfected using TransIT-LT1 reagent (Mirus, Madison, WI, USA) with 200 ng reporter plasmid DNA and 150 ng EGR1 or MAML1 expression vector DNA, as indicated in the figures. The cells were lysed in Reporter Lysis buffer (Promega, Madison, WI, USA) after 24 h and luciferase activity was measured using LucylSoft3. The data are reported as the mean values of at least three independent experiments.

Real-time PCR

Total RNA was purified using the RNaseasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and cDNA was synthesized using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7500 Sequence detector using the following primers sets: EGR1 forward, 5’-TACGAGCCCTGACCCGAG-3’; reverse, 5’-GACCAGACCTCTCTGTGGT-3’; 18S rRNA forward, 5’-CCTGCGGCTTTAATTTGACTG-3’; reverse, 5’-AGCTATCAATCTGTCAATCTTGTC-3’.

EGR1 mRNA expression was normalized to 18S rRNA in each sample.

In vivo acetylation assay

HEK-293 cells were transiently transfected with pcDNA3.1-FLAG-EGR1, pcDNA3.1-MAML1 and cmv-p300-HA using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA). After 24 h, the cells were treated with 10 mM sodium butyrate. The cells were harvested 48 h after transfection, lysed in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100 and complete EDTA-free protease inhibitor Pierce). Immunoprecipitation of FLAG-EGR1 was performed using M2-agarose that recognizes the FLAG epitope. The input and immunoprecipitation (IP) samples were analyzed by Western blotting using the following antibodies: EGR1 (Santa Cruz Biotechnology, CA, USA), the acetylated lysines in EGR1 (Cell Signaling Technology, Boston, MA, USA); p300 (Santa Cruz Biotechnology, CA, USA), MAML1 (Bethyl Laboratories, Cambridge, United Kingdom; Millipore, Billerica, MA, USA) and acetylated p300 lysine 1499 (Cell Signaling Technology, Boston, MA, USA).

Co-immunoprecipitation

Whole-cell lysates from HEK-293 cells were pre-cleared and endogenous MAML1 was immunoprecipitated using an MAML1 antibody (Santa Cruz Biotechnology, CA, USA). The input and IP samples were analyzed by Western blotting using the following antibodies: MAML1 (Bethyl laboratories, Cambridge, United Kingdom) and EGR1 (Santa Cruz Biotechnology, CA, USA).
Immunostaining

HEK293 cells were transfected with pcDNA3-FLAG-EGR1 and pcDNA3.1-MAML1 using TransIT-LT1 transfection reagent (Mirus, Madison, WI, USA), incubated for 24 h, washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA). The cells were immunostained using flag (Sigma-Aldrich, St. Louis, Missouri, USA) and MAML1 (Millipore, Billerica, MA, USA) antibodies, and analyzed using a Leica confocal microscopy (Leica Microsystems, Wetzler, Germany).

GST pull-down assay

HEK293 cells were transfected with pcDNA3.1-FLAG-EGR1 using TransIT-LT1 transfection reagent, and cultured for 2 h with 50 ng/ml TPA 24 h post-transfection. Whole cell lysates were prepared and 100 µl aliquots of protein extract in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 1 mM DTT and 1 x complete EDTA-free protease inhibitor cocktail) were incubated with GST proteins (prepared as described in [14]) bound to Glutathione Sepharose 4B beads (GE Healthcare, Little Chalford, United Kingdom) in 50 µl lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM KCl, 10% glycerol) overnight at 4°C. After washing with BC-150 buffer, the bound proteins were eluted in SDS-PAGE loading buffer (1 M Tris-HCl pH 6.8, 50% glycerol, 10% SDS, 1% bromophenol blue, 1 mM DTT), separated using 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes (GE Healthcare, Little Chalford, United Kingdom) and incubated with an antibody recognizing EGR1 (Santa Cruz Biotechnology, CA, USA).

Protein stability assay

HEK-293 FLAG-MAML1 and HEK-293 control cells were cultured in 24-well plates and treated with 50 ng/ml TPA for 2 h and 50 µg/ml cycloheximide (Sigma-Aldrich, St. Louis, Missouri, USA) for 1 h. The cells were harvested in lysis buffer (50 µM Tris-HCl pH 8, 150 mM NaCl, 3% Triton X-100) and complete EDTA-free protease inhibitor cocktail, the proteins were separated using SDS-PAGE and subjected to immunoblotting using an antibody recognizing EGR1 mRNA expression was analyzed by real-time RT-PCR. Transfection of HEK-293 cells with MAML1 siRNA or control siRNA, cultured with TPA for 2 hours, and EGR1 mRNA expression was analyzed by real-time RT-PCR. Transfection of HEK-293 cells with MAML1 siRNA significantly reduced the expression of EGR1 mRNA, compared to cells transfected with control siRNA (Figure 1C). In agreement with this observation, EGR1 mRNA increased in the MAML1-expressing cell line after induction with TPA, compared to control HEK-293 cells (Figure 1D). Thus, our data suggested that MAML1 might be involved in the regulation of EGR1 mRNA and protein expression in embryonic kidney cells.

Results and Discussion

MAML1 regulates EGR1 mRNA and protein expression

EGR1 protein stability has been reported to be stabilized by p300 acetylation, which results in the transactivation of survival genes [18]. As we previously found that MAML1 increases p300 autoacetylation, which enhanced the acetylation activity of p300 [11], we set out to investigate whether EGR1 expression levels could be elevated by MAML1. First, we transfected embryonic kidney (HEK-293) cells with MAML1 siRNA or control (Ctrl) siRNA, and cultured the cells in the presence of TPA to induce expression of EGR1. The proteins were detected by immunoblotting with antibodies recognizing MAML1, EGR1 and GAPDH. Expression of EGR1 was induced in cells cultured with TPA (Figure 1A, lanes 3 and 4); whereas the levels of EGR1 were reduced in cells treated with MAML1 siRNA (lane 4), compared to cells treated with control siRNA (lane 3). Next, we prepared whole cell extracts from HEK-293 cells stably expressing MAML1 and control HEK-293 cells, after inducing expression of EGR1 with TPA. The proteins were detected by immunoblotting with antibodies recognizing MAML1, EGR1 and GAPDH. As shown in Figure 1B, EGR1 was induced by TPA (lanes 2 and 4) and the expression of EGR1 significantly increased in cells overexpressing MAML1 (lane 4). Next, we investigated whether MAML1 affected EGR1 mRNA levels. HEK-293 cells were transfected with MAML1 siRNA or control siRNA, cultured with TPA for 2 hours, and EGR1 mRNA expression was analyzed by real-time RT-PCR. Transfection of HEK-293 cells with MAML1 siRNA significantly reduced the expression of EGR1 mRNA, compared to cells transfected with control siRNA (Figure 1C). In agreement with this observation, EGR1 mRNA increased in the MAML1-expressing cell line after induction with TPA, compared to control HEK-293 cells (Figure 1D). Thus, our data suggested that MAML1 might be involved in the regulation of EGR1 mRNA and protein expression in embryonic kidney cells.

EGR1 has a distinct spatio-temporal expression pattern during nephrogenesis [19], and disturbances in the regulation of EGR1 protein expression can lead to unwanted effects on normal cell fate. Overexpression of EGR1 has been reported to increase the proliferation of kidney cells and enhance tumorogenesis [21]. Therefore, proteins affecting the expression of EGR1, such as MAML1, might ultimately be connected to the EGR1 signaling pathway, which determines cell fate.

Bioinformatic analysis of MAML1, EGR1 and p300

Expression of MAML1, EGR1 and p300 were analyzed in tumor samples from 20 different cancer studies using the web-based cBio Cancer Genomics Portal (http://www.cbioportal.org/). last accessed 07/26/12) including genomics data, e.g., DNA copy number data from 5134 samples (array-comparative genomic hybridization) and mRNA expression data from 2430 samples (RNA-sequencing). Putative copy number alterations such as amplifications and homozygous deletions were derived within the portal using the “genomic identification of significant targets in cancer” algorithm [23]. The mRNA expression changes were considered significant if samples displayed a Z-score ≥2 compared to the reference population (diploid tumors or normal adjacent tissues if applicable). The association of altered gene expression with overall survival was assessed in selected cancer studies using Kaplan-Meier analysis. The patient samples were divided into two groups based on “gene set altered” or “gene set not altered” using the selected genomics data, and significant survival differences between the groups were determined using the log-rank test; p-values <0.05 were considered statistically significant.

MAML1 physically interacts with EGR1

We next investigated whether MAML1 could induce expression of an EGR1 target promoter, by transfecting the 4xEBS-luc reporter containing four EGR1 binding sites into cells expressing FLAG-MAML1 and control HEK-293 cells, and culturing the cells with TPA. The reporter activity increased when EGR1 was induced in control cells using TPA; however, the reporter activity was enhanced almost three fold in FLAG-MAML1 cells cultured with TPA, compared to control cells cultured with TPA (Figure 2A). In addition, HEK-293 cells were co-transfected with 4xEBS-luc and vectors expressing EGR1 and/or MAML1. While both EGR1 and MAML1 increased the activity of the EGR1 promoter; together EGR1 and MAML1 synergistically induced a 500-fold increase in the activity of the EGR1 target promoter (Figure 2B). Thus, our data suggest MAML1 may be recruited to the EGR1 signaling pathway, which determines cell fate.

To elucidate if MAML1 interacts with EGR1, whole-cell extracts were prepared from HEK-293 cells and MAML1 was immunoprecipitated using an antibody recognizing MAML1. The
proteins were separated by SDS-PAGE and Western blotting was performed using antibodies recognizing MAML1 and EGR1. As shown in Figure 2C, MAML1 and EGR1 interacted in the cellular extracts (lane 3). To determine whether EGR1 colocalized with MAML1, HCT116 cells were co-transfected with FLAG-EGR1 and MAML1 vectors and immunostained with antibodies recognizing FLAG and MAML1. EGR1 and MAML1 were strongly colocalized in the nucleus (Figure 2D). We noticed that the expression pattern of EGR1 in the nucleus changed in the presence of MAML1. We, and others, have previously reported that MAML1 colocalize MAML1-interacting proteins to nuclear bodies [24]. Our data suggest that EGR1 and MAML1 may form part of a transcription enhancer complex, which can increase the expression of genes with EGR1 binding sites. Both MAML1 and EGR1 are endogenously expressed in embryonic kidney cells, and both of these transcription factors have been shown to regulate gene expression during developmental processes [25,26]; however, it remains to be elucidated whether MAML1 and EGR1 cooperate to regulate any of these genes.

MAML1 and EGR1 synergistically activate the p300 promoter

The p300 promoter has been reported to contain EGR1 binding sites and to be regulated by EGR1 [18]. Therefore, we co-transfected HEK-293 cells with a p300-luc reporter and vectors expressing EGR1 and MAML1, and then cultured the cells in the presence of TPA. EGR1 alone activated the p300-luc reporter in the presence of TPA, and co-transfection of MAML1 with EGR1 strongly increased the activity of the p300 reporter (Figure 3A). We hypothesized that MAML1 may regulate the expression of p300, as well as the acetylation activity of p300 [11]. Lysates were prepared from a FLAG-MAML1 cell line and HEK-293 control cells, and the levels of p300 were analyzed by Western blotting using an antibody recognizing p300. The expression of p300 increased in the MAML1-expressing cell line, compared to control cells (Figure 3B, compare lane 2); whereas the expression level of GAPDH, which is not a MAML1 target, was similar in both cell lines (lanes 1 and 2).

EGR1 is a substrate for acetylation by p300 [18], and we have previously shown that MAML1 regulates p300 activity by increasing p300 autoacetylation [11]. Therefore, we investigated whether MAML1 regulates p300-dependent acetylation of EGR1.
Whole-cell extracts were prepared from HEK-293 cells and EGR1 was immunoprecipitated using an antibody recognizing EGR1. The proteins were separated by SDS-PAGE and Western blotting was performed using antibodies recognizing EGR1, the acetylated lysines in EGR1, MAML1, p300 and the acetylated lysine at 1499 of p300 (a marker of p300 autoacetylation). We noticed that expression of EGR1 increased in the presence of co-expressed MAML1 and/or p300 (Figure 3C, compare lane 1 to lanes 2–4). However, we only detected acetylated EGR1 in the presence of co-expressed p300 (lanes 3 and 4), which strongly increased in the presence of MAML1 (lane 4). The increased expression of EGR1 protein in the presence of co-expressed MAML1 (lane 2) might be due to MAML1-dependent up-regulation of EGR1 mRNA (see Figure 1). In agreement with our earlier observations, p300 autoacetylation was significantly increased by MAML1 (compare lanes 3 and 4). Thus, we presume that autoacetylated p300 acetylates EGR1 more efficiently.

Numerous reports have described how acetylation may increase protein stability [27]. Therefore, we induced expression of EGR1 using TPA in cells stably expressing MAML1 and HEK-293 control cells. After 1 h incubation with cycloheximide, significantly more EGR1 protein was present in the MAML1-expressing cell line compared to control cells (Figure 3D). Thus, we suggest that MAML1 may be involved in regulating the stability of EGR1, possibly by increasing the acetylation of EGR1. Acetylation of EGR1 by p300 has been reported to increase the protein levels of EGR1, to stimulate expression of cell growth and survival genes such as FGF2, PDGFB, TGFB1 and IGF2, and hence plays a crucial role in prostate cancer [15,18]. As EGR1 is expressed at high levels in prostate cancer and also certain renal cancers [21,22], it remains to be investigated whether p300-dependent acetylation of EGR1 affects the expression of EGR1 target genes or plays a role in the development of renal cancer.

The N-terminus of MAML1 cooperates with EGR1 during transcriptional activation

The MAML1 protein contains various domains, which are important for protein interactions and the function of MAML1 (see schematic in Figure 4A). Amino acids 1–74 of MAML1 control cells.
interact with the Notch ICD and MEF2C, while amino acids 75–305 are important for the interaction with p300. The N-terminal domain of MAML1 also interacts with p53 and GSK3β [24]. To investigate which MAML1 domain is required for activation of the promoters regulated by EGR1, plasmids expressing EGR1 and various MAML1 domains were co-transfected with the 4xEBS-luc reporter, which contains four EGR1 binding sites, into HEK-293 cells. As shown in Figure 4B, MAML1(1–1016) and (75–1016) strongly enhanced luciferase reporter gene activity in the presence of EGR1, while MAML1(1–625) had no detectable effect on EGR1-promoter activation. MAML1 (1–1016) and (75–1016) were expressed at similar levels in the cell culture assay, while we detected higher levels of MAML1(1–625) (Figure 4C).

In order to examine which region of MAML1 interacts with EGR1, full-length GST-tagged MAML1 protein and MAML1 derivatives (Figure 4D) were incubated with whole cell extracts from HEK293 cells transfected with EGR1. EGR1 interacted strongly with full-length GST-MAML1 protein and MAML1(1–300); but only showed a week interaction with MAML1(1–625) and (309–625) (Figure 4E). Since we detected a strong EGR1-interaction especially with MAML1(1–300), we further investigated the role of the N-terminus MAML1 in EGR1 transcription by co-transfecting HEK-293 cells with the 4xEBS-luc reporter and plasmids expressing EGR1, MAML1 full-length and MAML1(1–127). As shown in Figure 4F, MAML1(1–127) repressed the MAML1-EGR1 synergistic activation of the EGR1-promoter. We conclude that a domain within amino acids 75–127 of MAML1 may be important for the synergistic effect of MAML1 and EGR1 on gene transcription. The MAML1 N-terminus is reported to interact with Notch, MEF2C, p53 and GSK3β, and to play an important role in the transcriptional activity of these factors [1,2,5,6,13]. The N-terminal domain of MAML1 is also required for the interaction of MAML1 with p300, which leads to increased p300 autoacetylation and HAT activity [12]. In addition, MAML1 contains two N-terminal SUMOylation sites (K217 and K299) which repress MAML1 activity [14]. Therefore, the interactions of
MAML1 should be further investigated, in order to determine whether the interaction of EGR1 with MAML1 occurs in competition, or perhaps in synergy, with other transcription factors such as Notch and p53.

MAML1 positively correlates with EGR1 and p300 in kidney cancer

Based on the strong mechanistic evidence presented for co-regulation between MAML1, EGR1 and p300, we assessed a genomics database for such relationship. Our bioinformatic analysis of copy-number alterations (including amplifications and homozygous deletions) in the MAML1, EGR1 and p300 gene set revealed alterations in 18 of the 20 cancer studies assessed (Figure 5A). The highest level of alteration for the gene set was observed in renal clear cell carcinoma patients (7%) and renal papillary cell carcinoma patients (4%). Around 3% of the liver hepatocellular carcinoma and serous ovarian carcinoma patients displayed copy-number alterations for the gene set, while the other 14 cancer types showed alterations around 2% or below (Figure 5A). In contrast, no significant alterations were seen for pancreatic and prostate adenocarcinoma. Further analyses of the copy number changes in renal clear cell carcinoma showed that MAML1 was amplified in 7% of the samples and EGR1 was amplified in 5% of the samples (Figure 5B). MAML1 and EGR1 co-amplifications were observed in 61% of the samples from patients with either MAML1 or EGR1 amplification (data not shown).

Analysis of the mRNA expression levels extracted from the Cancer Genomics Portal (see Materials and Methods) indicated increased expression of MAML1, EGR1 and p300 in 5%, 7% and 4% of the renal clear cell carcinoma samples, respectively (Figure 5C). However, reduced expression of MAML1 and p300 were noted in 2% and 1% of the renal clear cell carcinoma samples, respectively. Expression changes in two or more genes from the MAML1, EGR1 and p300 gene set was observed in approximately 19% of the renal clear cell carcinoma samples (data not shown). The association between altered expression of the gene set at the mRNA level and overall survival in renal clear cell carcinoma was assessed. Kaplan-Meier analysis of the group of 69 patients with differential expression of at least one gene from the gene set (gene set altered) and the group of 349 patients with no
alterations (gene set not altered) revealed altered gene expression had a significant impact on overall survival (Figure 5D). Alterations in the gene set were associated with increased overall survival in renal clear cell carcinoma (p = 0.000356) compared to the unaltered gene set. Similar analysis revealed that copy-number alterations had no significant effect on overall survival (data not shown).

In conclusion, we provide evidence that the transcriptional coactivator MAML1 acts cooperatively with EGR1 to increase transcriptional activity of the EGR1 and p300 promoters in embryonic kidney cells. Moreover, using public data sets we have determined that alterations in the MAML1, EGR1 and p300 gene set were associated with increased overall survival in renal clear cell carcinoma (p = 0.000356) compared to the unaltered gene set. Similar analysis revealed that copy-number alterations had no significant effect on overall survival (data not shown).

In conclusion, we provide evidence that the transcriptional coactivator MAML1 acts cooperatively with EGR1 to increase transcriptional activity of the EGR1 and p300 promoters in embryonic kidney cells. Moreover, using public data sets we have determined that alterations in the MAML1, EGR1 and p300 gene set were associated with increased overall survival in renal clear cell carcinoma (p = 0.000356) compared to the unaltered gene set. Similar analysis revealed that copy-number alterations had no significant effect on overall survival (data not shown).

Acknowledgments

We thank Dr. G. Thiel and Dr. T.E. Elling for generously providing plasmids.

Author Contributions

Conceived and designed the experiments: MLH SB AEW. Performed the experiments: MLH SB SM. Analyzed the data: MLH SB RC SM GP RG BF AEW. Wrote the paper: SB RC AEW. Designed and performed the in silico (bioinformatics) experiments: RC RG.
References

1. Kitagawa M, Oyama T, Kawashima T, Yehrbohnick B, Kumar A, et al. (2001) A human protein with sequence similarity to Drosophila mastermind coordinates the nuclear form of notch and a CSL protein to build a transcriptional activator complex on target promoters. Mol Cell Biol 21: 4337–4346.

2. Wu L, Aster JC, Blacklow SC, Lake R, Artavanis-Tsakonas S, et al. (2000) MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet 26: 484–489.

3. Kurooka H, Honjo T (2000) Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. J Biol Chem 275: 17211–17220.

4. Oswald F, Tauber B, Dohner T, Bourteel S, Kostezka U, et al. (2001) p300 acts as a transcriptional coactivator for mammalian Notch-1. Mol Cell Biol 21: 7761–7774.

5. Shen H, McElhinny AS, Cao Y, Gao P, Liu J, et al. (2006) The Notch coactivator, MAML1, functions as a novel coactivator for MEF2C-mediated transcription and is required for normal myogenesis. Genes Dev 20: 675–688.

6. Zhao Y, Katzman RB, Delmolino LM, Bhat I, Zhang Y, et al. (2007) The notch regulator MAML1 interacts with p35 and functions as a coactivator. J Biol Chem 282: 11969–11981.

7. Alves-Guerra MC, Ronchini C, Capobianco AJ (2007) Mastermind-like 1 Is a specific coactivator of beta-catenin transcription activation and is essential for colon carcinoma cell survival. Cancer Res 67: 8090–8098.

8. Fryer CJ, Lamar E, Turbachova I, Kintner C, Jones KA (2002) Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. Genes Dev 16: 1397–1411.

9. Saint Just Ribeiro M, Hansson ML, Wallberg AE (2007) A proline repeat domain in the Notch co-activator MAML1 is important for the p300-mediated acetylation of MAML1. Biochem J 404: 289–298.

10. Wallberg AE, Pedersen K, Lendahl U, Roeder RG (2002) p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. Mol Cell Biol 22: 7812–7819.

11. Hansson ML, Popko-Scibor AE, Saint Just Ribeiro M, Lindberg MJ, et al. (2009) The transcriptional coactivator MAML1 regulates p300 autoacetylation and HAT activity. Nucleic Acids Res 37: 2996–3006.

12. Popko-Scibor AE, Lindberg MJ, Hansson ML, Holmlund T, Wallberg AE (2011) Ubiquitination of Notch1 is regulated by MAML1-mediated p300 acetylation of Notch1. Biochem Biophys Res Commun 416: 300–306.

13. Saint Just Ribeiro M, Hansson ML, Lindberg MJ, Popko-Scibor AE, Wallberg AE (2009) GSK3beta is a negative regulator of the transcriptional coactivator MAML1. Nucleic Acids Res 37: 6691–6700.

14. Lindberg MJ, Popko-Scibor AE, Hansson ML, Wallberg AE (2010) SUMO modification regulates the transcriptional activity of MAML1. FASEB J 24: 2396–2404.

15. Giterman D, Baron VT (2009) Is EGR1 a potential target for prostate cancer therapy? Future Oncol 5: 993–1003.

16. Liu C, Rangnekar VM, Adamson E, Mercola D (1998) Suppression of growth and transformation and induction of apoptosis by EGR-1. Cancer Gene Ther 5: 3–28.

17. Yu J, Baron V, Mercola D, Mustelin T, Adamson ED (2007) A network of p73, p53 and Eg1 is required for efficient apoptosis in prostate cells. Cell Death Differ 14: 436–446.

18. Yu J, de Belle I, Liang H, Adamson ED (2004) Coactivating factors p300 and CBP are transcriptionally crossregulated by Eg1 in prostate cells, leading to divergent responses. Mol Cell 15: 83–94.

19. Rackley RR, Kessler PM, Campbell C, Williams BR (1995) In situ expression of the early growth response gene-1 during marine nephrogenesis. J Urol 154: 708–705.

20. Pode-Shakked N, Dekel B (2011) Wilms tumor: a renal stem cell malignancy? Pediatr Nephrol 26: 1535–1543.

21. Scharnhorst V, Menke AL, Attema J, Haneyeveld JK, Riteco N, et al. (2000) EGR-1 enhances tumor growth and modulates the effect of the Wilms’ tumor 1 gene products on tumorigenicity. Oncogene 19: 791–800.

22. Strefford JC, Staesich V, Lane TM, Lu YJ, Oliver T, et al. (2005) A combination of molecular cytogenetic analyses reveals complex genetic alterations in conventional renal cell carcinoma. Cancer Genet Cytogenet 159: 1–9.

23. Beroukhim R, Getz G, Nghiemphu L, Barretina J, Hsu E, et al. (2007) Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. Proc Natl Acad Sci U S A 104: 20007–20012.

24. Saint Just Ribeiro M, Wallberg AE (2009) Transcriptional mechanisms by the coregulator MAML1. Curr Protein Pept Sci 10: 570–576.

25. McElhinny AS, Li JL, Wu L (2008) Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. Oncogene 27: 5138–5147.

26. Silverman ES, Collins T (1999) Pathways of Egr-1-mediated gene transcription in vascular biology. Am J Pathol 154: 665–670.

27. Lee KK, Workman JL (2007) Histone acetyltransferase complexes: one size doesn’t fit all. Nat Rev Mol Cell Biol 8: 284–295.