Genomic analysis of the role of transcription factor C/EBPδ in the regulation of cell behaviour on nanometric grooves

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ABSTRACT

C/EBPδ is a tumour suppressor transcription factor that induces gene expression involved in suppressing cell migration. Here we investigate whether C/EBPδ-dependent gene expression also affects cell responses to nanometric topology. We found that ablation of the C/EBPδ gene in mouse embryonal fibroblasts (MEFs) decreased cell size, adhesion and cytoskeleton spreading on 240 nm and 540 nm nanometric grooves. ChIP-SEQ and cDNA microarray analyses demonstrated that many binding sites for C/EBPδ, and the closely related C/EBPβ, exist throughout the mouse genome and control the upregulation or downregulation of many adjacent genes. We also identified a group of C/EBPδ-dependent, trans-regulated genes, whose promoters contained no C/EBPδ binding sites and yet their activity was regulated in a C/EBPδ-dependent manner. These genes include signalling molecules (e.g. SOCS3), cytoskeletal components (Tubb2, Krt16 and Krt20) and cytoskeletal regulators (ArhGEF33 and Rnd3) and are possibly regulated by cis-regulated diffusible mediators, such as IL6. Of particular note, SOCS3 was shown to be absolutely required for efficient cell spreading and contact guidance on 240 nm and 540 nm nanometric grooves. C/EBPδ is therefore involved in the complex regulation of multiple genes, including cytoskeletal components and signalling mediators, which influence the nature of cell interactions with nanometric topology.

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1. Introduction

C/EBP proteins form a highly conserved family of leucine zipper (bZIP) transcriptional factors that serve as master regulators of cellular processes such as the cell cycle, differentiation, and inflammatory responses [1]. So far, six C/EBP genes have been isolated (α, β, γ, δ, ε, and ζ), although protein numbers may well be higher due to variation in polypeptide size through alternate splicing and protein processing [2]. C/EBP isoforms are structurally similar, displaying a characteristic basic leucine zipper domain at the C terminus (90% homology between isoforms), which facilitates dimerization and DNA binding [2]. However, C/EBP isoforms are functionally and genetically distinct, with their transcriptional activation domains less well conserved (<20% sequence identity between isoforms). This divergence gives rise to the wide range of cellular responses in which C/EBP isoforms have been implicated [2].

Attention has recently turned to the functional role of the C/EBP isoform, which represents a ubiquitously expressed transcriptional activator that is robustly induced in a variety of G0 growth arrested cells [3]. Importantly, “loss of function” alterations in C/EBPδ have been reported in breast cancer [4–6] and acute myeloid leukaemia (AML) [7] and are generally associated with impaired contact inhibition, increased genomic instability and increased cell migration [8]. The use of knockout mice demonstrated that C/EBPδ has a vital role in mammary duct and epithelial cell proliferation [9] as well as an obligate role in the differentiation of preadipocytes [10,11]. In addition, recent work suggests that C/EBPδ is required for the maintenance of pluripotency in human limbic stem cells [2]. C/EBPδ activity appears to be regulated at a number of levels, including transcriptional (gene induction by STAT3, Sp1, CREB and NcoA/SRC-1 [12,13]), post-transcriptional (mRNA stability [14]) and post-translational (ubiquitinylation [15] and SUMOylation [16]) mechanisms. Certain C/EBP isoforms have also been shown to be substrates for various protein kinases, including the MAP kinases,
Fig. 1. Morphology of wild type (WT) and C/EBPδ−/− mouse embryonic fibroblasts (MEFs) cultured on flat or nanogrooved substrates. a) MEF WT and MEF C/EBPδ−/− cells were cultured on flat or nanogrooved substrates as indicated and stained for actin cytoskeleton (red) and vinculin (green). MEF WT cells had a well developed actin cytoskeleton and a largely polygonal morphology and became bipolar when cultured on grooves. In contrast C/EBPδ−/− MEFs had a less well developed actin cytoskeleton, were generally smaller and became very elongated when grown on the grooved substrate. b) MEF WT and MEF C/EBPδ−/− cell area and vinculin intensity were quantified as described in Materials and Methods. Results demonstrated that MEF C/EBPδ−/− cells were smaller and had lower levels of vinculin, on both flat and grooved surfaces, than MEF WT cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
ERK 1 and 2, and protein kinase C (PKC) [1], and are targets of second messenger signalling pathways. For example, elevations in the intracellular levels of cyclic AMP and activation of protein kinase A, has a direct impact on the induction of the constitutively active C/EBPβ isoform, which, in turn, regulates the acute-phase plasma protein gene haptoglobin, which is involved in the intestinal epithelial cell response to inflammation [12], whereas cyclic AMP-activation of expression protein activated by cyclic AMP 1 (EPAC1), leads to the C/EBPβ-dependent induction of the anti-inflammatory suppressor of cytokine signalling 3 (SOCS3) gene in vascular endothelial cells [17].

Despite being a transcriptional activator, very few C/EBPβ target genes have been identified. As a result the mechanisms by which C/EBPβ controls cell adhesion, migration, differentiation and cell-cycle progression remain poorly understood. Recent reports have demonstrated that sumoylation of C/EBPβ promotes sequestration to the nuclear periphery, thereby suppressing expression of C/EBPβ-dependent genes associated with cell adhesion, including glycoprotein V, proteocalcin 9 and integrin β8 [8,16]. Given this potential link between transcriptional control and cell adhesion we have used genonic analysis (including gene array and high resolution DNA sequencing) to investigate the role of C/EBPβ in controlling cell adhesion with biomaterials, comparing planar and nanometric grooved growth surfaces. 2. Materials and methods

2.1. Materials

Wild type (WT) immortalized mouse embryonic fibroblasts (129Sv/C57C16, MEFs) and MEFs containing homozygous deletions of the C/EBPβ, or C/EBPβ genes were generous gifts from Prof. Peter Johnson (C/EBPβ) and Esta Sterneck (C/EBPβ) from NCI, National Institutes of Health, Frederick, MD. SOCS3 and FAK knockout MEFs, together with matched WT cells, were gifts from Prof. Margaret Frame, Edinburgh Cancer Research UK Centre, UK and Dr Timothy Palmer, Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK, respectively. Forskolin, and rolipram were purchased from Merck Biosciences (Nottingham, UK). Anti-SOCS3 and ChIP-grade, anti-C/EBPβ polyclonal antibodies were from Santa Cruz. 2.2. Generation of grooved and planar growth surfaces

Microstructured quartz substrates were fabricated by acid-cleaning (7:1H2SO4:H2O2 for 5 min) quartz slides which were then spin-coated with AZ primer (4000 rpm for 30sec). A layer of Shipley 5181 photoresist (Shipley) was spun onto the spin-coated slides and then soft-baked for 30 min in a 90°C oven. The substrates were UV treated on a MA6 mask aligner with exposure energy of ca. 71 mJ/cm² per second in hard contact, through an electron-beam fabricated chrome mask with 12.5 µm wide lines. The resist was developed (1:1 AZ developer (Microchemicals)/water) for 65sec. The substrates were rinsed, dried and then etched in a trichloromethane environment at a rate of 25 nm/min in a reactive ion etching unit (RIE80, Plasma Technology) using the polymer pattern as an etch resist to generate 240 or 540 nm deep grooves. The residual resist was removed with acetone and the slide was then cleaned with ethanol for a further 1 min to produce a homogeneous surface chemistry. Planar slides were then blanket etched to ensure that the chemistry was comparable with the structured substrates. Quartz substrates were then cleaned in Caro’s acid solution (2:1 H2SO4:H2O2 for 20 min, rinsed six times with distilled water and air-dried under a Class 1 or II sterile flow hood. Imprints of the quartz substrates into polycaprolactone (PCL) were achieved by hot-embossing, the resulting imprints, 240 nm and 540 nm grooves, were trimmed for use and planar PCL (Ra of 1.7 nm over 10 µm) was used as a control substrate. The PCL samples were given a 30 s treatment in oxygen plasma to allow cell attachment (Harrick Plasma, USA). All PCL substrates were sterilised in ethanol for 30 min, then transferred through three rinses in sterile 1× PBS and two rinses in complete medium.

2.3. Cell culture and growth of cells on biomaterials

Matched wild type (WT) or homozygous knock-out (–/–) mouse embryonic fibroblasts (MEFs), for C/EBPβ, C/EBPβ, SOCS3 or FAK, were maintained in 71% (v/v) DMEM (Sigma), supplemented with 17.7% (v/v) medium 199, 9% (v/v) FBS, 1% (v/v) M-199 (RIE80, Plasma Technology) using the polymer pattern as an etch resist to generate chemicals):water) for 65 sec. The substrates were rinsed, dried and then etched in the mask with 12.5 µm features, and the medium was replaced regularly. Unless otherwise indicated, MEFs were seeded at a density of 1 × 10⁶ cells/ml on the PCL-tumbled-embossed substrates with a 24 h culture period. 2.4. Immunofluorescence

MEFs were fixed in a 10% (v/v) formaldehyde solution (15 min at 37°C, per- methylated (5 min at 4°C) and blocked in 1% (v/v) BSA/PBS (15 min at 37°C). The samples were then stained at 37°C for 1 h with 1:200 (v/v) anti-vinculin (clone hvin-1, Sigma) in 1% (w/v) BSA/PBS and 1:500 (v/v) phallolidin-rodilinoid (Molecular Probes). In each experiment, two replicas each of planar and nano groove topography were stained. Cells were washed 3 × 5 min in 1×PBS containing 0.5% (v/v) Tween, and appropriate biotinylated secondary antibody (Vector Laboratories) was added at 1:50 in 1% (w/v) BSA/PBS and incubated for a further hour at 37°C. After washing, 1:50 (v/v) FITC-conjugated streptavidin (Vector Laboratories) was added to the samples and incubated for 30 min at 4°C followed by washing and mounting using Vectashield mounting medium with DAPI nuclear stain (Vector Laboratories). 2.5. Microarray analysis

C/EBPβ WT, C/EBPβ–/–, C/EBPβ WT and C/EBPβ–/– cells were grown on planar growth surfaces and then incubated in the presence or absence of a combination of 10 µM forskolin plus 10 µM rolipram (F/R) for 5 h at 37°C in 5% (v/v) CO2. Cells were then washed with 1 × 1 ml PBS and RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturers protocol. RNA samples were additionally treated with a “DNA-free Kit” (Applied Biosystems) to remove any remaining DNA. RNA samples were then prepared for Affymetrix whole transcriptome microarray analysis, using the WT Expression Kit (Ambion) according to the manufacturer’s instructions. Briefly, reverse transcription was used to prime poly(A) and non-poly(A) mRNA and generate sense strand cDNA for fragmenta- tion and labelling, using the Affymetrix GeneChip WT Terminal Labeling Kit (PN 900671). Amplified and biotinylated sense-strand DNA targets were hybridised, using a Fluidic Station 400, to Affymetrix GeneChip Mouse Gene 2.0 ST Array and data captured using an Affymetrix GeneChip Scanner 3000 7G. Gene expression changes were selected where at least one fold change was greater than 2. Grouping of gene expression data into similarly responsive patterns was done using Treeview and CLUSTER software [18], freely available from the Eisen lab (http://www.eisenlab.org/). 2.6. Chromatin immunoprecipitation and sequencing (ChIP-SEQ) analysis

C/EBPβ WT and C/EBPβ WT cells were stimulated in the presence or absence of a combination of 10 µM forskolin plus 10 µM rolipram (F/R) for 5 h at 37°C in 5% (v/v) CO2. Cells were then fixed and chromatin extracted and sheared using the enzymatic “CHIP-IT Express Kit” (Active Motif) according to the manufacturer’s instructions. Sheared chromatin from F/R-treated and non-treated cells was then immunoprecipitated at 4°C, overnight with 4 µg of either C/EBPβ WT or C/EBPβ ChIP-grade antibodies (Santa Cruz). DNA fragments were eluted from immunopre- cipitated chromatin and used to prepare a ChIP-SEQ DNA library for sequencing using the “CHIP-SEQ Sample Prep Kit” from Illumina, according to the manufacturer’s protocols. Briefly, the first step in library preparation was to convert any overhangs in the ChIP’d DNA into phosphorylated blunt ends. The 3’ ends were then adenylation and adaptors ligated onto the ends of the fragments. The library was then size selected on an agarose gel and eventually enriched by PCR. The enriched library samples were then loaded onto a flow cell at a concentration of 12µM and cluster formation was done on an Illumina Cluster station. Samples were then sequenced on an Illumina GA IIx giving 76bp reads.

2.7. ChIP-SEQ data analysis

The CHIP DNA was sequenced on an Illumina GA IIx, one lane of the flow cell per sample. The quality of the reads was assessed using Fastqc (www. bioinformatics.babraham.ac.uk/projects/fastqc/). The sequence reads were aligned to the mouse genome (release version mm9) using the bowtie aligner (version 0.12.7) [bowtie.bio.sourceforge.net/index.shtml] [19], which was set up to report only uniquely aligning reads. Duplicate reads were removed using Samtools (version 0.1.18) [20]. The ChIP analysis was performed using the Homer (version 3.5) suite of tools (biowhat.ucsd.edu/homer/ngs/index.html) [21]. The pipeline for the analysis is shown in Supplementary Fig. 1. Custom scripts were created to compare the list of known genes closest to ChIP peaks with gene lists generated from RNA microarray experiments. These corroborated gene lists were visualised on chromosomes using Circos plots (circos.ca; Supplementary Fig. 2) and Venn diagram (Fig. 2b).

2.8. Reverse transcription PCR (RT-PCR)

Total RNA isolation was extracted from MEF WT and MEF–/– cells using a RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. RT-PCR reactions were carried out using the OneStep RT-PCR Kit from Qagen. Briefly, 5–10 ng of RNA per sample was used in 25 ul reaction mixture containing 0.4 μM
Validation of C/EBPβ ChIP-Seq and microarray

### Validation of C/EBPβ ChIP-Seq

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<th>log P-value</th>
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<th>% of Background</th>
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Validation of C/EBPδ ChIP-Seq

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Fig. 2. Results of C/EBPβ and C/EBPδ ChIP-SEQ and microarray. a) MEF WT cells were incubated in the presence or absence of a combination of the cyclic AMP-elevating agents, forskolin and rolipram (F/R). Following stimulation cells were fixed, chromatin was isolated and then immunoprecipitated (ChIP) with anti-C/EBPβ or C/EBPδ antibodies, as described in Materials and methods. ChIP DNA samples were then sequenced (ChIP-SEQ) on an Illumina GA IIx DNA sequencer. ChIP analysis of the resulting DNA sequences was then performed using the Homer (version 3.9) suite of tools (biohut.ucsd.edu/homer/ngs/index.html). The figure shows part of the Homer analysis indicating that aligned sequences from each ChIP experiments contained bone fide C/EBP consensus binding motifs, hence validating the experimental technique. b) MEF WT, MEF C/EBPβ−/− and MEF C/EBPδ−/− cells were stimulated for 5 h in the presence or absence of F/R. Cells were then harvested, RNA isolated, biotinylated sense-strand DNA synthesised and hybridised to mouse whole genome GeneChip® ST Arrays (Affymetrix). A Venn diagram was then generated to illustrate the degree of overlap between genes identified by ChIP-SEQ as containing consensus, F/R-dependent C/EBP-binding sites and F/R-induced changes in MEF gene expression.

3. Results and discussion

3.1. The role of C/EBPδ in controlling cell adhesion and spreading on nanometric grooves

In order to determine the role of C/EBPδ transcription factor in regulating cell interaction with nanometric topology, embryonal fibroblasts isolated from wild type (MEF WT), or transgenic mice in which both alleles encoding C/EBPδ had been deleted (MEF C/EBPδ−/−), were grown on planar or nanogroove (240 nm or 540 nm depth, fixed 12.5 μm groove/ridge width (25 μm pitch)) surfaces.
Fig. 3. Gene expression changes from microarray that are also identified by C/EBP ChIP-SEQ microarray analysis was carried out on RNA samples isolated from MEF WT, MEF C/EBP \( ^b \)/C0/\( ^C \) and MEF C/EBP \( ^d \)/C0/\( ^C \) cells that had been stimulated in the presence or absence (CNTRL) of F/R for 5 h. Gene expression ratios from all cell types were then matched with genes identified from ChIP-SEQ as containing C/EBP binding sites. CLUSTER analysis was then used to generate a dendrogram (on the left) to group together gene changes with similar expression profiles. The enlarged sections on the right represent groups of genes that are either induced (blue) or repressed (yellow) following F/R treatment and display a dependency for either C/EBP \( ^b \), C/EBP \( ^d \) or both, as represented by a directional change in gene expression in samples isolated from either MEF C/EBP \( ^b \)/C0/\( ^C \) or MEF C/EBP \( ^d \)/C0/\( ^C \) cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Gene expression changes from microarray that are also identified by C/EBPδ ChIP-SEQ. CLUSTER analysis was carried out on gene expression ratio changes that were also identified by C/EBPδ ChIP-SEQ, as described in the legend to Fig. 3. Enlarged elements of the CLUSTER dendogram on the right represent groups of genes whose responsiveness to F/R treatment is dependent on the expression of either C/EBPδ, C/EBPβ, or both.
Fig. 5. Gene expression changes from microarray that are also identified by both C/EBPβ and C/EBPδ ChIP-SEQ CLUSTER analysis was carried out on microarray gene expression changes that were also identified by ChIP-SEQ as genes with consensus binding sites for both C/EBPβ and C/EBPδ ChIP.

Cells were then fixed and immunostained with anti-actin and anti-vinculin antibodies (Fig. 1a). From these experiments it was apparent that MEF WT cells spread normally on planar growth surfaces, possessing a well-developed actin cytoskeleton and adhesion complexes, as indicated by phalloidin and vinculin staining respectively. In addition, MEF WT cells grown on 240 nm or 540 nm grooves became more elongated and aligned themselves along the direction of the nanogrooves (Fig. 1a). In contrast, MEF C/...
Fig. 6. Gene expression changes from microarray that were not identified by either C/EBPβ or C/EBPδ ChIP-SEQ. CLUSTER analysis was carried out on microarray gene expression changes on genes that were not identified by either C/EBPβ or C/EBPδ ChIP-SEQ. These gene identities probably represent genes that are either regulated by F/R, independently of C/EBPβ or C/EBPδ, or genes that are trans-activated by C/EBPβ or C/EBPδ.
EBPδ cells grown on a flat substrate were more rounded, with a poorly developed actin cytoskeleton and reduced focal adhesions, as demonstrated by a reduction in vinculin staining (Fig. 1b). MEF C/EBPδ cells also appeared more stellate with extensive arborisation and, although they underwent contact guidance on nanometric grooves, became much more elongated and were much less spread than MEF WT cells, particularly on 540 nm grooves. These results demonstrate that the transcription factor C/EBPδ plays an essential role in efficient adhesion and spreading to both planar and nanometric grooved surfaces. These results are consistent with a recent report demonstrating that MEF C/EBPδ cells display increased migration when compared with MEF WT cells in wound healing assays [22] and suggests that C/EBPδ may play a role in regulating the expression of genes involved in cell adhesion, movement and spreading.

3.2. Identification of C/EBPδ consensus binding sites within the mouse genome

In order to test whether C/EBPδ does in fact regulate gene expression associated with interactions with cell growth surfaces we carried out genomic analysis to identify the full range of genes and gene promoters regulated by C/EBPδ in MEFs. We stimulated MEF WT and MEF C/EBPδ cells for 5 h with a combination of the pharmacological agents (F/R), forskolin, which promotes cyclic AMP synthesis, and rolipram, which inhibits cyclic AMP degradation, to increase the intracellular levels of C/EBPδ protein through the activation of the C/EBPδ gene [17] (Fig. 8). As a comparison, and to ensure that we could identify genes specifically regulated by C/EBPδ, C/EBPβ WT and −/− cells were treated in a similar fashion. This was done because many of the cellular actions of C/EBPδ are shared by C/EBPβ [17]. Following stimulation MEF WT cells were fixed, lysed and cellular chromatin isolated, fragmented and immunoprecipitated (ChIP) with anti-C/EBPδ or anti-C/EBPβ antibodies. The genomic DNA associated with ChIPd samples was then sequenced using a genome analyser (ChIP-SEQ) to identify the C/EBPδ are C/EBPβ binding sites throughout the genome. The sequence reads were aligned to the mouse genome using the Bowtie aligner (version 0.12.7), which was set up to report only uniquely aligning reads, and duplicate reads were removed using Samtools (version 0.1.18). The Homer (version 3.9) suite of tools was used to verify that the majority of reads contained bone fide C/EBP-binding sites (Fig. 2a).

To complement the ChIP-SEQ analysis, mRNA was also extracted from F/R-treated MEF C/EBPδ WT, MEF C/EBPδ −/−, MEF C/EBPβ WT and MEF C/EBPβ −/− cells. The extracted mRNA was then converted by reverse transcription to cDNA probes, which were hybridised to mouse whole genome microarrays (Affymetrix). This approach was used so as to determine whether the genes that contain C/EBP-binding sites, identified by ChIP-SEQ, are also regulated at the level of transcription in a C/EBP-dependent manner.

Fig. 7. RT-PCR analysis demonstrates that C/EBPδ is required for the regulation of genes encoding cytoskeletal components and IL6 MEF WT or MEF −/− cells were incubated for 5 h with a combination of forskolin and rolipram (F/R), following which RNA was extracted, reverse transcribed and PCR amplified using the indicated primers. Results demonstrate that F/R regulates the expression of mRNAs encoding cytoskeletal components (a) and IL6 (b) in a C/EBPδ-dependent manner.

Fig. 8. Deletion of the C/EBPδ gene in MEFs blocks the induction of SOCS3 mRNA and protein MEF WT and MEF C/EBPδ −/− cells were stimulated for 5 h with F/R to elevate intracellular cyclic AMP levels. This resulted in the induction of C/EBPδ and SOCS3 protein in MEF WT, but not MEF C/EBPδ −/−, cells as detected by Western blotting in the upper panel. Deletion of the C/EBPδ gene also blocked the ability of F/R to induce SOCS3 mRNA expression, as detected by RT-PCR (lower panel).
Genes identified as being regulated by F/R, or by deletion of either C/EBPβ or C/EBPδ, are listed in Supplementary Data Set 2. Custom scripts were then created to compare the list of known genes closest to ChIP peaks with gene lists generated from RNA microarray experiments (Supplementary Data Set 1). These corroborated gene lists were visualised on chromosomes using Circos plots (circos.ca; Supplementary Fig. 2) and a Venn diagram (Fig. 2b). From these analyses we identified over 1100 genes that interact with C/EBPβ alone and a similar number that interact with C/EBPδ alone (Fig. 2b; Supplementary Data Set 1). Over 650 genes contain binding sites for both C/EBPβ and C/EBPδ (Fig. 2b; Supplementary Data Set 1). Generally, C/EBPβ and C/EBPδ binding sites were distributed evenly throughout the cellular chromosomes (Supplementary Fig. 2). Of the approximate 7800 genes identified by cDNA microarray as being regulated by the F/R treatment regime (Supplementary Data Set 2), over 500 were identified as containing C/EBPβ binding sites, over 500 contained C/EBPδ binding sites and over 170 contained binding sites for both C/EBPβ and C/EBPδ.

Fig. 9. Morphology of Wild Type (WT) and SOCS3 −/− MEFs Cultured on Flat or Nanogrooved Substrates MEF WT and MEF SOCS3 −/− cells were cultured on flat or nanogrooved substrates and stained for actin cytoskeleton (red) and vinculin (green). It can be seen in (a) that C/EBP −/− MEFs were smaller and had a poorly developed actin cytoskeleton and were unable to contact align to nanogrooved substrates. MEF WT and MEF SOCS3 −/− cell area (b) and vinculin intensity (c) were quantified as described in Materials and Methods. Results demonstrated that MEF SOCS3 −/− cells were smaller and had lower levels of vinculin (c) on nanogrooved surfaces, than MEF WT cells. Western blotting with anti-SOCS3 antibodies (d) demonstrated that MEF WT cells also expressed significantly lower levels of SOCS3 protein on 540 nm nanometric grooves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
These figures far exceed the approximate 100 C/EBP\textsubscript{d} target genes previously identified through ChIP–chip assays in a previous study [8] and demonstrates the sensitivity of our combined ChIP-SEQ and cDNA microarray approach. Taken together our data demonstrates that C/EBP\textsubscript{b} and C/EBP\textsubscript{d} isoforms interact promiscuously with the mouse genome through direct interaction multiple C/EBP-binding sites. Moreover, the fact that C/EBP\textsubscript{d} specifically interacts with over 1000 genes, independently of C/EBP\textsubscript{b}, suggests that C/EBP\textsubscript{d} may play a unique and specific role in the control of cell behaviour through the independent regulation of multiple gene regulatory networks.

3.3. Identification of genes whose expression is specifically regulated by C/EBP\textsubscript{d}

Results this far indicate that there are many regulatory C/EBP\textsubscript{d} binding sites distributed throughout the mouse genome. Accordingly, the identities of genes that contain C/EBP\textsubscript{d} binding sites are diverse and don’t immediately describe shared functions (Supplementary Data Set 1). The same can be said for C/EBP\textsubscript{b}-interacting genes (Supplementary Data Set 1). Therefore, in order to further delineate the function of C/EBP\textsubscript{d} in MEFs we carried out CLUSTER analysis [18] on the F/R-induced gene expression changes that were identified by both cDNA microarray and SEQ analysis (Supplementary Data Set 2). CLUSTERS were generated for gene expression changes associated with C/EBP\textsubscript{b} (Fig. 3), C/EBP\textsubscript{d} (Fig. 4) and shared (C/EBP\textsubscript{b} plus C/EBP\textsubscript{d}; Fig. 5) ChIP-SEQ and therefore represent genes that are potentially cis-regulated by C/EBPs \textsubscript{b} and/or \textsubscript{d}. From these results in can be seen that F/R stimulation effects the induction or suppression of a large number of genes, where the direction of change is similar between MEF WT and MEF \textsubscript{C/EBP\textsubscript{b}}/\textsubscript{C/EBP\textsubscript{d}}/\textsubscript{C/EBP\textsubscript{d}} cells (Figs. 3–5). This indicates that although C/EBPs \textsubscript{b} and/or \textsubscript{d} physically interact with these genes, they do not appear to be required for transcriptional regulation by F/R. In contrast, CLUSTER analysis did reveal a number of gene groups (the enlarged portions of Figs. 3–5) that share a common expression patterns characterised by a directional change in gene expression relative to MEF WT cells, in one or both of MEF C/EBP\textsubscript{b} \textsubscript{--} and/or MEF C/EBP\textsubscript{d} \textsubscript{--} cells, indicating a dependency on C/EBP\textsubscript{b} and/or C/EBP\textsubscript{d} for the expression change. However, examining the majority of gene identities from C/EBP\textsubscript{d}-dependent CLUSTERS, it is not immediately clear.
which genes might be directly responsible for the effects of C/EBPβ on cell adhesion, migration and spreading that we observed on planar and nanometric growth surfaces (Fig. 1). We did note, however, that the RHO guanine nucleotide exchange factor 33 (ARHGEF33) appeared to be up-regulated in F/R-stimulated C/EBPβ –/− cells (Fig. 4), indicating a possible link between C/EBPβ and the regulation of the tubulin cytoskeleton.

Surprisingly we could not find any other C/EBPβ-regulated, cytoskeleton linked gene identity in either of the C/EBPβ or δ ChIP-SEQ lists, apart from RHO-family GTPase 3 (RND3), which was associated with C/EBPβ ChIP, and hence is likely to be trans-, rather than cis-, activated by C/EBPβ. Consistent with a trans-activation role for C/EBPβ, we did find a number of genes encoding cytoskeleton components in Fig. 6 (tubulin β2 (TUBB2), Keratin 16 (KRT16) and Keratin 20 (KRT20)), which details a CLUSTER of gene expression changes that do not match with ChIP-SEQ and therefore represent genes that are possibly trans-regulated by C/EBPβ and/or δ. Trans-regulation by C/EBPβ could occur through either the induction of expression of additional, “tertiary”, transcription factor(s) or through the activation of autocrine/paracrine signalling mechanisms. Interestingly, in this respect, F/R treatment was found to up-regulate the expression of the gene encoding the autocrine/paracrine cytokine IL6. Of these newly identified elements, such as C/EBPβ/C0, we identified a general repressor of RND3 gene activity (Fig. 7a). Basal levels of RND3 levels were enhanced in MEF C/EBPβ –/− cells, whereas actin levels were unchanged, demonstrating that C/EBPβ is a general repressor of RND3 gene activity (Fig. 7a).

Together these results demonstrate that C/EBPβ plays a central role in the regulated expression of cytoskeleton components (TUBB2, KRT16 and KRT20) and cytoskeleton regulators (ARHGEF33 and RND3) in MEFS. Since no direct interaction was detected between C/EBPβ and the genes encoding these cytoskeletal components, it is likely that an intermediary, trans-activating element, such as C/EBPβ-regulated IL-6 (Fig. 7b), may be required for their regulation. These observations may go some way to explain the requirement for C/EBPβ for proper adhesion and spreading on planar and nanometric growth surfaces described in Fig. 1.

3.4. The role of SOCS3 in spreading and contact guidance on nanometric grooves

Another potential target for IL6-mediated, C/EBPβ-dependent trans-activation is the suppressor of cytokine signalling 3 (SOCS3) gene. SOCS3 is known to be a direct target of IL6/Receptor activator of nuclear factor kappa-B (NF-kB) and hence is likely to be trans-activated, rather than cis-activated, by C/EBPβ. Consistent with a trans-activation role for C/EBPβ, we did find a number of genes encoding cytoskeleton components in Fig. 6 (tubulin β2 (TUBB2), Keratin 16 (KRT16) and Keratin 20 (KRT20)), which details a CLUSTER of gene expression changes that do not match with ChIP-SEQ and therefore represent genes that are possibly trans-regulated by C/EBPβ and/or δ. Trans-regulation by C/EBPβ could occur through either the induction of expression of additional, “tertiary”, transcription factor(s) or through the activation of autocrine/paracrine signalling mechanisms. Interestingly, in this respect, F/R treatment was found to up-regulate the expression of the gene encoding the autocrine/paracrine cytokine IL6, which is a C/EBPβ-interacting gene whose expression was further enhanced in MEF C/EBPβ –/− cells (Fig. 4). We confirmed this observation by RT-PCR analysis of IL6 mRNA levels in C/EBPβ WT and C/EBPβ –/− cells (Fig. 7b) and contrasted these effects with the expression levels of the transcription factor nuclear receptor (NR) 4a3 and the signal transduction intermediate, insulin receptor substrate (IRS) 1, whose mRNA levels were either induced or repressed, respectively, following F/R treatment, but were unaffected by knockout of the C/EBPβ gene (Fig. 7b). Moreover, RT-PCR analysis confirmed that the genes encoding TUBB2, RND3, KRT16 and KRT20 were also induced by F/R treatment, consistent with a potential intermediary role for IL6, however only Krt20 was the only cytoskeletal component to be further induced following C/EBPβ deletion, whereas TUBB2 and KRT16 expression was suppressed (Fig. 7a). Basal levels of RND3 levels were enhanced in MEF C/EBPβ –/− cells, whereas actin levels were unchanged, demonstrating that C/EBPβ is a general repressor of RND3 gene activity (Fig. 7a).

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Appendix A. Supplementary data

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References


