

## Review

# The Many Faces of C/EBP $\delta$ and their Relevance for Inflammation and Cancer

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 Corresponding author: Email: sternecg@mail.nih.gov Phone: +1-301-846-1471; Fax: +1-301-846-1666.© Ivyspring International Publisher. This is an open-access article distributed under the terms of the Creative Commons License (<http://creativecommons.org/licenses/by-nc-nd/3.0/>). Reproduction is permitted for personal, noncommercial use, provided that the article is in whole, unmodified, and properly cited.

Received: 2013.07.22; Accepted: 2013.08.27; Published: 2013.09.20

## Abstract

The CCAAT/enhancer binding protein delta (CEBPD, C/EBP $\delta$ ) is a transcription factor that modulates many biological processes including cell differentiation, motility, growth arrest, proliferation, and cell death. The diversity of C/EBP $\delta$ 's functions depends in part on the cell type and cellular context and can have opposing outcomes. For example, C/EBP $\delta$  promotes inflammatory signaling, but it can also inhibit pro-inflammatory pathways, and in a mouse model of mammary tumorigenesis, C/EBP $\delta$  reduces tumor incidence but promotes tumor metastasis. This review highlights the multifaceted nature of C/EBP $\delta$ 's functions, with an emphasis on pathways that are relevant for cancer and inflammation, and illustrates how C/EBP $\delta$  emerged from the shadow of its family members as a fascinating "jack of all trades." Our current knowledge on C/EBP $\delta$  indicates that, rather than being essential for a specific cellular process, C/EBP $\delta$  helps to interpret a variety of cues in a cell-type and context-dependent manner, to adjust cellular functions to specific situations. Therefore, insights into the roles and mechanisms of C/EBP $\delta$  signaling can lead to a better understanding of how the integration of different signaling pathways dictates normal and pathological cell functions and physiology.

Key words: C/EBP, transcription factor, cell signaling, inflammation, cancer.

## Introduction

The *CEBPD* gene encoding the C/EBP $\delta$  protein, a member of the C/EBP transcription factor family, was cloned by sequence homology more than 20 years ago. Several reviews have been written about the C/EBP family and have emphasized other C/EBP proteins [1-6]. This article will focus specifically on C/EBP $\delta$ . Initially, C/EBP $\delta$  was mostly used as a marker for adipocyte or myeloid differentiation and of inflammatory activation. Early work on C/EBP $\delta$  was conducted primarily in cell lines and attributed a variety of functions to C/EBP $\delta$  that were not necessarily fully supported by studies *in vivo*. For example, C/EBP $\delta$  is not *essential* for adipocyte or myeloid differentiation in mice. Intriguingly, studies of C/EBP $\delta$  in different systems have led to it being labeled as a "tumor suppressor" or also as a tumor-promoting "mesenchymal master regulator" [7, 8]. From these seeming contradictions, and from more recent studies using *Cebpd* knockout mice, it has become apparent

that C/EBP $\delta$  in fact functions as a modulator of many processes. This review summarizes and attempts to integrate some of our current knowledge about C/EBP $\delta$ , with an emphasis on findings that have relevance for cancer biology and inflammatory signaling.

Normal cellular functions occur through the orchestrated temporal and spatial interactions of molecules, whose roles have evolved and been selected for over millions of years. Genetic mutations and epigenetic changes that disturb these interactions may result in dedifferentiation, loss of growth control, or escape from cell death mechanisms, and eventually lead to cancer. Most cancer-related deaths are due to metastatic disease, which is the result of a series of complex steps in which malignant cells disseminate from the primary tumor and begin to establish growth in other organs [9]. It is also now apparent that most cancers can thrive only by corrupting the normal host

environment, at both the cellular microenvironment and systemic levels [10].

Proteins whose aberrant activity can drive tumor development are termed oncogenes. Proteins whose normal activity primarily inhibits tumor development have been termed tumor suppressors. However, since the emergence of the “TGF- $\beta$  paradox” [11, 12], it has become ever more clear that this nomenclature is often inaccurate or, at best, incomplete, and that this limitation in conceptualizing protein functions applies not only to cancer but to most paradigms in cell and organismal biology. Indeed, such labels can hinder our appreciation of the full complexity of molecular functions and of the diversity of molecular interactions that underlie the great variety of cellular and physiological outcomes. Our current knowledge of C/EBP $\delta$  suggests that it is one of the molecules that defy labeling, because it takes on multiple, context-dependent functions. The detailed study of such molecules presents an opportunity to gain deeper insights into the diversity and complexities of signaling pathways and their adaptations that govern normal and pathological conditions.

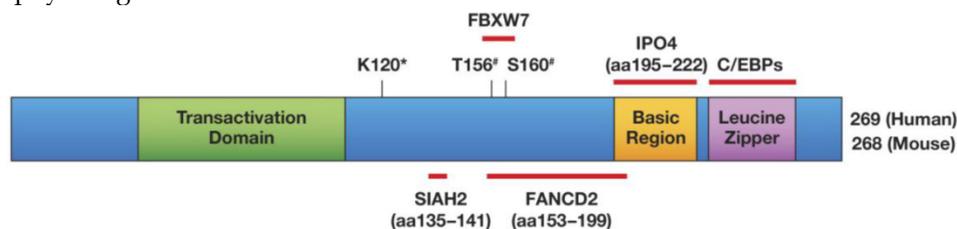
### C/EBP $\delta$ as a versatile modulator of gene transcription

The C/EBP family of transcription factors is defined by a highly homologous basic region-leucine zipper (bZIP) domain that mediates its dimerization and DNA binding. C/EBP $\delta$  is encoded by the single-exon *CEBPD* gene, and only one full-length protein isoform is known to date. C/EBP factors can bind DNA only as dimers by virtue of the leucine zipper scissors’ grip mechanism [1]. C/EBP $\delta$ ’s confirmed heterodimerization partners consist of all the other C/EBP proteins, including C/EBP $\zeta$ /CHOP as well as ATF4 [13]. However, the specific roles of such heterodimeric interactions in C/EBP $\delta$ ’s physiological functions are completely unknown. Interestingly, both CHOP and the ATFs are stress response factors, with well-characterized roles in hypoxia and the unfolded protein response [14]. These proteins are prime candidates for physiological heterodimer formation

with C/EBP $\delta$ , especially under hypoxia (see section below). This notion awaits further investigation.

C/EBP proteins exhibit largely identical DNA-binding specificities, at least *in vitro* [2, 13, 15]. Therefore, it is impossible to predict from DNA sequences which C/EBP member may target a particular gene. For example, the aromatase promoter is activated by C/EBP $\beta$  but not by C/EBP $\delta$  in human adipose fibroblasts [16]. Furthermore, target gene regulation may also depend on interactions with other regulatory factors. The prolactin gene promoter is repressed by C/EBP $\delta$  and activated by C/EBP $\alpha$ , in each case through interaction with Pit-1 [17, 18]. Comprehensive surveys of C/EBP $\delta$ ’s target genes by chromatin immunoprecipitation (ChIP) followed by subsequent validation using cells with C/EBP $\delta$  knockdown or knockout have been reported for a mouse mammary epithelial cell line [19], human keratinocytes [20], and mouse macrophages [21]. In addition, C/EBP $\delta$  ChIP data from K562 and HepG2 cells are now available from the ENCODE project (encodeproject.org).

C/EBP $\delta$  has a *bona fide* transcription transactivation domain [1], and most of C/EBP $\delta$ ’s target gene promoters are activated by it [19-21]. **Figure 1** illustrates C/EBP $\delta$ ’s protein structure, highlights its protein interactions that have been confirmed with endogenous proteins, and shows the amino acids that are required for functional modifications. Target gene transactivation can be achieved by C/EBP $\delta$ ’s interaction with the CBP co-activator: that is, C/EBP $\delta$  triggers CBP phosphorylation, which depends on residues Leu81 and Phe82 on C/EBP $\delta$  [22]. However, C/EBP $\delta$  can also inhibit gene expression, depending in part on its protein modification or selective protein interactions. Sumoylation of C/EBP $\delta$ , which requires its K120 residue, abolishes its interaction with the CBP/p300 co-activator, and may be one mechanism by which C/EBP $\delta$  becomes a transcription repressor instead of an activator [23]. The K120 residue is also required for the acetylation of C/EBP $\delta$  by p300/CBP *in vitro* and may contribute to target gene transactivation [23, 24].



**Fig 1. Schematic of the C/EBP $\delta$  protein, and mapped modifications and interactions.** Only interactors that have been mapped to a sub-domain of C/EBP $\delta$  and whose interactions were confirmed at the level of endogenous proteins have been included in this Figure: SIAH2 [56], FBXW7 [57], FANCD2 [41], IPO4 [41]. Amino acids (aa) that are important for protein modifications are indicated: #phosphorylation by GSK-3 $\beta$ ; \*K120 is necessary for acetylation and sumoylation [23] and for ubiquitination by SIAH2 [56] but not by SCF<sup>FBXW7</sup> [57]. K120 was not confirmed as the acceptor site for these modifications. Transactivation Domain: aa 41-108 [60, 178] Basic Region/Leucine Zipper: aa 195-222/226-254 [6].

The physical interaction of C/EBP $\delta$  with SP1 [25] or ZNF638 [26] leads to synergistic gene activation, whereas C/EBP $\delta$ 's interactions with Rad [27] or DIPA [28] inhibit the activation of target genes. C/EBP $\delta$  represses the expression of IGF-1 in osteoblasts [29], CD-RAP in chondrocytes [30], apoC-III in hepatoma cells [31, 32], manganese superoxide dismutase [33], and FBXW7 $\alpha$  [34]. C/EBP $\delta$ 's interaction with HDAC1 contributes to the repression of the haptoglobin [35] and of the PPAR $\gamma$  (PPARG) promoter [36], whereas its interaction with FOXO3a activates PPAR $\gamma$  expression [37]. C/EBP $\delta$ 's association with GATA4 [38] or Runx 2 [39] can modulate target gene activation versus repression. Furthermore, its interaction with SMAD3 inhibits the DNA-binding function of C/EBP $\delta$ , which may be part of the mechanism by which TGF- $\beta$  inhibits adipocyte differentiation [40]. Although far from complete, the diverse effects illustrated in the above list suggest that almost anything can result from C/EBP $\delta$  binding to a target promoter. Furthermore, a non-transcriptional function for C/EBP $\delta$  has also been discovered: it chaperones the FANCD2 DNA repair protein into the nucleus [41]. FANCD2 has also been shown to interact with the transcription factor NF $\kappa$ B and to inhibit as well as activate gene expression [42, 43]. Therefore, it is quite plausible that FANCD2 also modulates target gene regulation by C/EBP $\delta$ , in addition to the role of their interaction in nuclear translocation of FANCD2.

Most of the above-mentioned data were generated in cultured cell lines. Insights into physiologically relevant functions have been derived from mice with a null mutation of the *Cebpd* gene. Analysis of *Cebpd* knockout (KO) mice revealed that the protein is not essential for normal development or the adult life of mice in a pathogen-free environment [44, 45]. This is consistent with C/EBP $\delta$ 's expression being low to undetectable in most organs and cell types. However, C/EBP $\delta$  expression is rapidly but transiently induced by a variety of stimuli [1, 6], which may be indicative of C/EBP $\delta$ 's function as a modulator for specific physiological adaptations. **Table 1** summarizes the phenotypes that have been characterized in *Cebpd* null mice to date.

## Regulation of C/EBP $\delta$ expression and activity

As mentioned above, C/EBP $\delta$  expression is typically low to undetectable. However, a number of extracellular signals, including cytokines, bacterial lipopolysaccharide (LPS), and corticosteroid, can induce it, suggesting that C/EBP $\delta$  has a role in stress responses [6]. Hypoxia also induces C/EBP $\delta$  in cultures

of various cell types, and in certain tissues, including brain and tumors [7, 34, 47, 48]. **Table 2** summarizes some of the reports on the regulation of C/EBP $\delta$  expression, and highlights cases in which inflammatory regulators and hormones induce its expression. In addition, Runx factors have been described as either activating or inhibiting the *CEBPD* promoter activity [39, 49]. Finally, C/EBP $\delta$  can activate its own gene expression [1, 21].

Induced C/EBP $\delta$  expression is typically transient. In macrophages, LPS induces *CEBPD* through NF $\kappa$ B, which is subsequently silenced by ATF3 [21]. This feedback loop does not exist in kidney fibroblasts, where *CEBPD* is induced through c-Jun and maintained by a mechanism requiring IRAK-1 [50]. Interestingly, the histone deacetylase inhibitor trichostatin A interrupts c-Jun's binding to SP-1 and the *Cebpd* promoter, and prevents LPS-induced C/EBP $\delta$  expression in RAW264.7 macrophages [51]. Thus, *CEBPD* expression can also be modulated through modification of the c-Jun protein. In keratinocytes, *CEBPD* gene expression is inhibited by  $\Delta$ Np63 $\alpha$ , while C/EBP $\delta$  augments  $\Delta$ Np63 $\alpha$  expression, providing a mechanism for the transient C/EBP $\delta$  induction during differentiation [52]. The transient nature of C/EBP $\delta$  expression hints that persistently elevated C/EBP $\delta$  levels might lead to an adverse outcome. This concept will be discussed in more detail below, in the section on Inflammation.

At the level of *mRNA stability*, C/EBP $\delta$  expression can be increased by the binding of HuR to its 3'UTR. This mechanism can be activated by UV radiation through p38MAPK, and by prostaglandin E2 (PGE<sub>2</sub>), which trigger the nuclear to cytoplasmic translocation of HuR [53, 54]. Ectopic microRNA let-7c downregulates the *CEBPD* mRNA level in macrophages by a mechanism that requires the 3'UTR, which suggests that the *CEBPD* mRNA is directly targeted by let-7c [55].

At the level of *protein stability*, C/EBP $\delta$  is downregulated by the SIAH2 E3 ubiquitin ligase [56] and the SCF-FBXW7 $\alpha$  polyubiquitination complex [57]. SIAH2 can act as an oncogene [58], while FBXW7 $\alpha$  is a tumor suppressor in epithelial cancers [59]. Hence, two potentially antagonizing pathways both downregulate C/EBP $\delta$  expression. In turn, C/EBP $\delta$  inhibits the expression of FBXW7 $\alpha$  [34], resulting in a negative feedback loop [57]. The degradation of C/EBP $\delta$  by FBXW7 depends on C/EBP $\delta$ 's prior phosphorylation on threonine T156 (and possibly serine S160) by GSK-3 $\beta$  kinase. C/EBP $\delta$  is therefore stabilized by signals that inhibit GSK-3 $\beta$  activity, such as TLR4 activation [57].

**Table 1: Summary of C/EBP $\delta$  knockout mouse phenotypes.** Only reports with *in vivo* studies (i.e., not those using only cultured cells) are included.

Primary organ and cell type(s)	Main loss of function phenotype	Affected Gene(s)#	Strain background	References
Adipocytes	Exacerbated impairment of adipocyte differentiation due to C/EBP $\beta$ null mutation	<i>Ucp1</i> ↓	Mixed C57BL/6x129/Ola	[44]
Adipocytes	Reduced body and fat mass index specifically in females*	-	Mixed 129S1xC57BL/6 (F2)	[81]
CNS, dendritic cells	Less severe clinical score for experimental autoimmune encephalomyelitis	<i>Il10</i> ↑	C57BL/6	[126]
Brain, glia	Reduced LPS response	<i>Tnfa</i> ↓ <i>Il6</i> ↓ <i>Il1b</i> ↓	C57BL/6	[127]
Brain	Enhanced learning by contextual fear conditioning	-	Mixed 129S1xC57BL/6	[45]
Peripheral motor neurons	Impaired regeneration after sciatic nerve injury	<i>Spr1a</i> ↓	C57BL/6	[163]
Tumor implant, lymphendothelial cells	Impaired lymphangiogenesis in grafted tumors	<i>Vegfc</i> ↓ <i>Vegfr3</i> ↓	C57BL/6	[48]
Systemic inflammatory, macrophages	Decreased collagen-induced arthritis	<i>Ccl20</i> ↓ <i>Cxcl1</i> ↓ <i>Il23a</i> ↓ <i>Tnfaip6</i> ↓	C57BL/6	[164]
Systemic, inflammatory	Reduced LPS responses and increased survival	Systemic: <i>Il-6</i> ↓ <sup>***</sup> <i>TNF<math>\alpha</math></i> ↓	C57BL/6	[122]
Systemic, macrophages	Impaired clearance, specifically of high titer <i>E. coli</i> infections	<i>Il6</i> , <i>Saa3</i> , <i>Cxcl2</i> and others↓	C57BL/6	[21]
Lung, macrophages	Reduced macrophage infiltration and clearance of <i>Klebsiella</i> infection**	Systemic <i>Il-6</i> ↑ <i>TNF-<math>\alpha</math></i> ↑	C57BL/6	[75]
Lung, epithelial and inflammatory cells	Relative resistance to pneumococcal pneumonia**	<i>Pafr</i> ↓	C57BL/6	[120]
Lung, macrophages	Reduced LPS-induced lung injury	Lung: <i>Il-6</i> , <i>TNF-<math>\alpha</math></i> , <i>MIP-2</i> ↓	C57BL/6	[82]
Kidney	Reduced kidney damage after renal ischemia/reperfusion injury**	Systemic <i>TNF<math>\alpha</math></i> ↓ <i>Il-6</i> ↓	C57BL/6	[122]
Kidney, tubular cells	Reduced renal absorption of lactate and urate	<i>Slc5a8</i> ↓ <i>Slc5a12</i> ↓	C57BL/6	[165]
Kidney, mesangial cells	Reduced experimental glomerulonephritis	<i>Acta2</i> ↓	129	[166]
Mammary gland	Delayed cell death and involution after lactation##	<i>Tp53</i> ↓ <i>Bcl2a1</i> ↓ <i>Igf1p5</i> ↓ <i>Ccnd1</i> ↑ Survivin↑	C57BL/6	[70]
Mammary gland, tumor	Increased MMTV-Neu mammary tumor multiplicity and reduced lung metastases, impaired hypoxia adaptation	<i>Fbxw7a</i> ↑ <i>Cxcr4</i> ↓	FVB/N	[34]
Mammary gland, tumor; peritoneal macrophages	Reduced LPS response	<i>Fbxw7a</i> ↑ <i>Tlr4</i> ↓	FVB/N	[57]
Mammary gland	Increased ductal branching in aged mice ***	-	Mixed 129S1xC57BL/6	[90]
<b>Negative Results:</b>				
Ovary, theca/interstitial cells	Not essential despite LH-induced expression	-	129S1	[71]
Bone	Normal bone density	-	Mixed 129S1xC57BL/6 (F2)	[81]
Mammary gland	Morphologically normal involution##	<i>Trpm2</i> ↓ <i>Igf1p5</i> ↓	Mixed 129S1xC57BL/6	[90]
Lung, macrophages	Normal inflammatory response to IgG	-	C57BL/6	[83]

#This column highlights some of the genes/proteins whose expression is regulated directly or indirectly by C/EBP $\delta$ . See citations for details.

## Note contradictory results of [90] and [70], possibly due to strain background.

\*This phenotype was not observed previously in a study using the same strains [45], nor in studies using mice backcrossed to C57BL/6 [70], 129S1 [71], or FVB/N strains [34]. We speculate that the reduced body mass of C/EBP $\delta$  null female mice may be due to a maternal/paternal effect, because only this study [81] used compound C/EBP $\delta$  and C/EBP $\beta$  heterozygous breeders.

\*\* Controls were not from the same cross as mutants.

\*\*\*This phenotype was not observed in mice backcrossed to the C57BL/6 or FVB/N strain background ([70], and unpublished data) and may therefore be specific to the mixed strain background.

**Table 2: Regulation of C/EBP $\delta$  expression:** Compilation of some reports describing activators or inhibitors of C/EBP $\delta$  expression at the RNA and/or protein level. See text for additional examples and further discussion. *Exogenous Agent:* Experimental treatments. *Mediator(s):* Molecules that regulate C/EBP $\delta$  expression directly or indirectly by pharmacological inhibition/activation, overexpression, RNA interference, or gene deletion. Parentheses indicate inferred mediators. *Cell type(s):* Predominant cell type used in the cited studies (h, human; m, mouse; r, rat; MEF, mouse embryo fibroblast; VSMCs, vascular smooth muscle cells). *Associated Process:* Cellular response to agent/mediator(s) whether functional relevance for C/EBP $\delta$  in that process was demonstrated or not. *Reference(s):* Examples are given with preference for most recent publications, as earlier literature on the subject is typically discussed in these papers.

Exogenous Agent	Mediator(s)	Cell type(s)	Associated Process	References
<b>Induction of C/EBP<math>\delta</math> expression</b>				
EGF	MAPK, CREB	A431 epidermoid carcinoma (h)	Growth Arrest	[167]
Hypoxia	HIF-1	lymphendothelial cells (h)	Lymphangiogenesis	[48]
LPS	NF $\kappa$ B (REL)	Bone marrow-derived macrophages (m)	Inflammatory signaling	[21]
LPS	IRAK1, AP-1	Kidney fibroblasts (m)	Chronic inflammation	[50]
LPS	IRAK1, IKK $\epsilon$ (IKBKE)	Bone marrow-derived macrophages (m), MEF (m)	Inflammatory signaling	[61, 118]
Testosterone		CWR22 prostate cancer cells	Proliferation/progression	[94]
Testosterone (DHT)	Androgen receptor	Rv1 and R1 prostate cancer cell lines (h)	?	[168]
Progesterone	Progesterone receptor B	MDA-MB-231 breast tumor cells (h)	Inhibition of proliferation	[169]
Dexamethasone	Glucocorticoid receptor	C <sub>2</sub> C <sub>12</sub> myotubes, muscle (m); A549 lung epithelial cells (h)	Muscle atrophy; glucocorticoid response	[132] [131]
IL-1	JNK, CK2, p50/p65 NF $\kappa$ B	Hep3B hepatoma (h)	Inflammatory signaling	[170]
IL-1 $\beta$ +IFN- $\gamma$	STAT1	INS-1E insulinoma cells (r)	Inflammation, cell death	[112]
IL-6	STAT3, SP1	HepG2 hepatoma cell line (h)	(Inflammatory signaling)	[171]
IL-6	STAT3	LNcaP prostate cancer cells (h)	Growth arrest	[92]
Oncostatin M	JAK2/STAT3	HC11 mammary epithelial cells (m)	Growth arrest	[88]
Insulin	PI3K	VSMC	Proinflammatory gene expression	[172]
	Runx2	Osteoblasts (r)	Proliferation	[39]
cAMP	Mdm2/CREB/Crtc2	MEF	Adipogenesis	[173]
Vasoactive intestinal peptide, pituitary adenylate cyclase activating protein, noradrenalin,	(cAMP)	Cortical astrocytes (m)	Glycogen synthesis	[174]
1-(2-hydroxy-5-methylphenyl)-3-phenyl-1,3-propanedione (HMDDB)	p38, CREB	A431 (epidermoid) and HeLa (cervical) carcinoma cell lines (h)	Cell death	[99]
1,25-Dihydroxyvitamin D3	(VDR)	LNcaP prostate cancer cells; MCF-7, T47D breast cancer cells (h)	Growth inhibition	[93]
<b>Inhibition of C/EBP<math>\delta</math> expression</b>				
M-CSF	Let-7c	Bone marrow-derived macrophages (m)	M2 polarization	[55]
Src (family) kinase	SIAH2	Breast tumor cell lines (h)	C/EBP $\delta$ degradation, "transformation"	[56]
	GSK3 $\beta$ , FBXW7 $\alpha$	Peritoneal macrophages	C/EBP $\delta$ degradation, inhibition of LPS- response	[57]
Hsp70	TLR2, IL-10	Bone marrow-derived dendritic cells	Anti-inflammatory	[175]
LPS	ATF3	Bone marrow-derived macrophages (m)	Feed-back inhibition of inflammatory signaling	[21]
LPS, TNF $\alpha$	Miz1(pS178)	Lung tissue and MLE-12 lung epithelial cells (m)	Attenuation/inhibition of inflammatory signaling	[135]
	Myc/Max/Miz1	HC11 mammary epithelial cells (m)	(proliferation)	[134]
Chrysin (Flavonoid)	?	Embryonic cortical neurons, microglia cultures (m), BV2 microglial cell line (m)	Inhibition of inflammation	[128]
Troglitazone	Inhibition of STAT3 by PPAR $\gamma$	VSMCs	Inhibition of IL-1 $\beta$ response	[136]
TGF- $\beta$	?	VSMC	Inhibition of IL-1 $\beta$ -induced expression	[162]
	SUZ12/YY1	A431 (epidermoid) and HeLa (cervical) carcinoma cell lines (h)	(proliferation, tumor progression)	[142]
	$\Delta$ Np63 $\alpha$	HaCaT cells and primary keratinocytes (h)	Keratinocyte differentiation	[52]
Pref-1	Sox9	MEF, 3T3-L1 pre-adipocytes	Inhibition of adipocyte differentiation	[176]
Tunicamycin	IRE $\alpha$	Liver, hepatocytes	Unfolded protein response, prevention of steatosis	[177]
Testosterone	(indirect)	Prostate tissue (r)	(tissue maintenance)	[94]

Although no other phosphorylation sites on C/EBP $\delta$  have been mapped to date, several additional reports point to a role of phosphorylation in the regulation of C/EBP $\delta$  activity. The serine residue S191 in the extended basic region of C/EBP $\delta$  is necessary for the PGE $_2$ -mediated activation of C/EBP $\delta$  in osteoblasts, although the kinase has not been identified, and S191 has not been confirmed as a phosphorylation site [60]. IL-1 treatment of HepG2 cells induces the tyrosine-phosphorylation of C/EBP $\delta$  [32], Casein Kinase II phosphorylates C/EBP $\delta$  *in vitro* [15], and IKKi/IKK $\epsilon$  (IKBKE) is essential for the LPS-induced phosphorylation of C/EBP $\delta$  [61]. In all cases, the phosphorylation events stimulated C/EBP $\delta$ 's DNA-binding activity [15, 32, 61]. Experiments with phosphatase inhibitors also support that C/EBP $\delta$  needs to be phosphorylated to bind DNA [62]. Future investigations will have to unravel how IL-1, LPS, CaKII, IKBKE, and also GSK-3 $\beta$  intersect to regulate the C/EBP $\delta$  pathway. In addition to phosphorylation, the C/EBP $\delta$  protein can be modified by acetylation and sumoylation. These modifications require an intact K120 lysine residue, and, as mentioned above, serve to switch its transactivator function on or off [23]. Interestingly, the same K120 lysine residue is also required for the ubiquitination and degradation of C/EBP $\delta$  by SIAH2 [56].

By virtue of nuclear localization signals in its DNA-binding domain, C/EBP $\delta$  is primarily a nuclear protein, which is translocated at least in part by the IPO4 importin [41]. However, C/EBP $\delta$ 's nuclear localization can be regulated. For example, TNF- $\alpha$  not only induces C/EBP $\delta$  expression, but also promotes its nuclear accumulation in the adult rat hepatocyte cell line RALA255-10G [63]. C/EBP $\delta$ 's nuclear translocation is augmented by the DNA-damaging agent mitomycin C [41]. Billiard et al. demonstrated that a flag-tagged C/EBP $\delta$  protein is constitutively nuclear in rat calvarial osteoblasts, but the endogenous protein is cytoplasmic in the absence of PGE $_2$  [64]. This last report highlights the potential for misconstruing physiological roles by relying too heavily on overexpression studies.

C/EBP $\delta$  constitutively resides in the nucleus of primary vascular smooth muscle cells (VSMCs) from the hypertensive rat, but not in those from normal rats [65]. In VSMCs, IL-1 $\beta$  triggers the relocation of C/EBP $\delta$  from its perinuclear localization to the nucleus [66]. The perinuclear localization of C/EBP $\delta$  has also been observed in a mammary epithelial cell line ectopically expressing the SUMO E3 ligase PIASy [67]. In differentiating adipocytes, during the clonal expansion phase, C/EBP $\delta$  localizes to the centromere when cells enter the S phase [68]. Thus, C/EBP $\delta$ 's

activity can be regulated at several levels by the modulation of its subcellular localization. However, the mechanistic details of these pathways and/or their physiological relevance remain to be determined.

Nuclear localization is not equivalent with activity. In a cell culture model of adipocyte differentiation, the DNA-binding activity of C/EBP $\delta$  is delayed compared to its induced expression [68]. In human primary osteoblasts, cytoplasmic PKA is necessary for the nuclear localization of C/EBP $\delta$ , while further activation of C/EBP $\delta$  requires nuclear PKA to mediate PGE $_2$ -induced IGF-1 promoter transactivation. However, PKA does not phosphorylate C/EBP $\delta$  *in vitro*, suggesting that the mechanism may be indirect [69].

Analyses of C/EBP $\delta$  expression in tissues have so far highlighted its primarily nuclear localization, which has been reported in the involuting mouse mammary gland [70], hypoxic/necrotic areas of mouse mammary tumors and human glioblastoma [7, 34], theca/interstitial cells of the mouse ovary [71], human breast epithelial cells [72], meningioma tumor cells [73], and lung bronchial epithelial and alveolar type II cells [74, 75]. However, even when specific, diffuse immunohistochemical staining in the cytoplasm can be difficult to distinguish from background, leaving open the possibility that C/EBP $\delta$  is more widely expressed in the cytoplasm than is currently recognized.

To conclude, much remains to be learned about how the various signaling pathways that regulate C/EBP $\delta$  expression and activity at different levels and through subcellular localization are integrated to control C/EBP $\delta$ 's functions and target gene responses.

### Integration of C/EBP $\delta$ signaling with other C/EBP family members

All five members of the C/EBP family can heterodimerize [13] and therefore could give rise to 15 different dimers with potentially unique or also redundant functions. The existence of several isoforms of C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\epsilon$  [1] may lead to even greater complexity. If and how this diversity is realized in nature is largely unknown. While many studies have described either synergistic or antagonistic regulation of expression of certain C/EBP family members, few have explored the functional consequences of such expression patterns. All full-length C/EBP proteins have been largely associated with growth arrest and differentiation functions [76]. However, these can be cell type-specific and exceptions exist, especially when considering different

isoforms of the same protein [3, 4, 76]. The subsequent sections will present examples for C/EBP $\delta$  that highlight its multifaceted nature.

As mentioned above, certain gene promoters may be regulated differentially by specific C/EBP factors in cultured cells. However, the *in vivo* relevance of these observations has not been established. Most compellingly, gene replacement studies in mice have illustrated that C/EBP $\beta$  can substitute for C/EBP $\alpha$  in hematopoiesis and adipocyte differentiation [77, 78]. However, the mitochondrial biogenesis in fat cells is significantly altered in such mouse mutants [78]. It is conceivable that in many cases, C/EBP factors have redundant functions and act synergistically as suggested for C/EBP $\beta$  and C/EBP $\delta$  in inflammatory signaling [79, 80] and adipocyte differentiation [44]. Variations in expression between C/EBP members could be merely due to the divergence in the regulation of expression while preserving similar functions. For example, the inducible C/EBP $\delta$  protein may support the role of C/EBP $\beta$  that exhibits a more constitutive expression pattern [81]. Furthermore, similar functions may have been channeled into different signaling pathways. For example, C/EBP $\beta$  but not C/EBP $\delta$  is essential for immune complex-induced lung injury in mice, whereas C/EBP $\delta$  - not C/EBP $\beta$  - mediates LPS-induced lung injury, although both proteins are induced by the treatments [82, 83]. On the other hand, antagonistic roles of C/EBP $\alpha$  and C/EBP $\delta$  have been observed with respect to HIF-1 $\alpha$  regulation. C/EBP $\alpha$  inhibits HIF-1 function by competing with HIF-1 $\alpha$  for HIF-1 $\beta$  binding [84]. In contrast, C/EBP $\delta$  enhances HIF-1 activity by supporting HIF- $\alpha$  expression [34]. If and how these opposing functions are utilized in the same cell to modulate cell biology has not been explored. To fully understand the role of C/EBP $\delta$  functions compared to other C/EBP members, more systematic functional comparisons will be necessary including in-cell analysis of heterodimers and gene replacement studies that allow for temporal and spatial control of expression.

### **C/EBP $\delta$ and the regulation of cell proliferation and differentiation**

C/EBP $\delta$  was first cloned by homology to other C/EBP factors and was subsequently recognized as a gene that is induced during growth arrest and the differentiation of certain cell types. For example, C/EBP $\delta$  expression is induced during G0 growth arrest, promotes contact inhibition in mammary epithelial cells [85, 86] and fibroblasts [87], and mediates the growth arrest of mammary epithelial cell lines in response to Oncostatin M [88]. However, the effect of C/EBP $\delta$  on proliferation can be cell-type dependent,

and may reflect its role in the expression of secreted factors and/or intracellular regulators of the cell cycle.

Ectopic C/EBP $\delta$  reduces the growth rate of mammary epithelial cell lines but not of HEK293 cells [89]. These findings are in agreement with the observation that the loss of C/EBP $\delta$  leads to hyperproliferation and excess branching of the mammary gland epithelium in aged mice of a mixed 129B6 strain background [90]. In cultured mammary and myeloid tumor cell lines C/EBP $\delta$  promotes the downregulation of cyclin D and/or cyclin E and p27CIP2 expression [85, 89, 91], which is consistent with the suppression of cell growth. C/EBP $\delta$  also mediates growth inhibition by IL-6 or 1,25-dihydroxyvitamin D3 in the androgen-dependent LNCaP prostate cancer cell line [92, 93]. In contrast, another study described high basal levels of C/EBP $\delta$  expression in androgen-dependent human prostate cancer cells (three primary lines and CWR22 cells), which are further enhanced by testosterone [94]. Thus, more studies are needed to clarify if C/EBP $\delta$  regulates prostate cancer cell growth or differentiation *in vivo*, and how its functions may be modulated by hormone-receptor signaling.

In addition to regulating the expression of intrinsic cell-cycle regulators, C/EBP $\delta$  also modulates cell proliferation and growth arrest indirectly through the expression of secreted factors. In pituitary prolactinoma cells, C/EBP $\delta$  inhibits proliferation by suppressing prolactin expression [18]. On the other hand, C/EBP $\delta$  promotes the proliferation of cultured osteoblasts by directly activating the expression of IGF-1 [95]. Similarly, C/EBP $\delta$  promotes the proliferation of VSMCs in response to IL-1 $\beta$  by inducing expression of the PDGFA-receptor [66]. These cases in which C/EBP $\delta$  stimulates proliferation indirectly through secreted factors demonstrate clearly that the expression of C/EBP $\delta$  does not necessarily inhibit cell proliferation and can be compatible with cell proliferation.

In addition to the regulation of proliferation, C/EBP $\delta$  has also been implicated in the differentiation of certain cell types. For example, C/EBP $\delta$  expression is associated with early stages of pre-adipocyte differentiation [96, 97]. The loss of C/EBP $\delta$  alone *in vivo* impairs the development of brown fat and further exacerbates an adipogenesis defect observed in C/EBP $\beta$  KO mice [44]. In addition, studies of adipogenic differentiation in cell culture have provided molecular evidence for a direct role of C/EBP $\delta$  in tumor suppressor pathways. That is, C/EBP $\delta$  (like C/EBP $\beta$  and C/EBP $\alpha$ ) specifically associates with the hypophosphorylated form of the retinoblastoma protein, Rb, which predominates in

the G0/G1 phase of the cell cycle [98]. For C/EBP $\beta$  it was determined that the interaction with Rb potentiates promoter transactivation and promotes adipocyte differentiation; this interaction must be regulated by additional factors or specific modifications, because it is only seen in differentiating cells [98]. On the other hand, Rb interferes with reporter gene transactivation by C/EBP $\delta$  in the HeLa and A431 cancer cell lines where C/EBP $\delta$  attenuates the activity of the Rb partner E2F-1 and promotes apoptosis [99]. In breast cancer cell lines, C/EBP $\delta$  promotes phosphorylation of Rb, which is associated with Rb's tumor suppressor function, through downregulation of the kinase inhibitor cyclin D1 [56]. In a BCR-ABL-positive cell line (KCL22), C/EBP $\delta$  also interacts with Rb and E2F-1 and induces growth arrest and myeloid differentiation [91]. In summary, while the reported details vary, C/EBP $\delta$ 's intersection with the Rb pathway has been correlated with cell death, differentiation, and inhibition of oncogenic transformation.

Consistent with these observations, a number of reports point to a tumor-suppressor function of C/EBP $\delta$  in the *myeloid* lineage. The *CEBPD* promoter is silenced in 35% of 260 human acute myeloid leukemia samples [8], and its expression is downregulated in the blast crisis phases of *chronic myelogenous leukemia* (CML) [100]. The overexpression of C/EBP $\delta$  in primary mouse hematopoietic progenitor cells or a cell line model of *acute myelogenous leukemia* (AML) leads to myeloid differentiation accompanied by upregulation of the G-CSF receptor and downregulation of c-myc expression [8, 101], as also seen in CML cell lines [91]. Indeed, C/EBP $\delta$  is expressed in differentiated granulocytes/neutrophils [91, 102]. In macrophages, C/EBP $\delta$  regulates many genes associated with functions of differentiated cells [21] and is specifically associated with M1 macrophage polarization [55]. While direct experimental evidence from mouse models is still missing, the potential tumor suppressor function of C/EBP $\delta$  in AML and possibly CML is most likely due to its function in stimulating differentiation, which may or may not be correlated with its function in cell cycle arrest.

The antithesis of the differentiated cell is the undifferentiated *stem cell*, with its self-renewal capacity. Very little information exists about C/EBP $\delta$  expression or functions in stem cells. One report describes C/EBP $\delta$  expression in limbal stem cells of the eye, where it maintains p63 expression, prolongs the cell cycle, and maintains stemness [103]. In addition, C/EBP $\delta$  expression was observed in cultured long-term self-renewing neural stem cells, dependent on the stem-cell maintenance factor LIF [104]. A role for C/EBP $\delta$  in stem-cell maintenance is consistent with its ability to downregulate drivers of the cell

cycle. Interestingly, FBXW7 $\alpha$ , which inhibits and is inhibited by C/EBP $\delta$  [34, 57], has been implicated in promoting stem-cell differentiation, pluripotency, and quiescence, depending on the cell type [105-107]. Therefore, the potential roles and mechanisms of C/EBP $\delta$  signaling in stem/progenitor cells are a promising but currently under-explored area of research.

## C/EBP $\delta$ and the regulation of cell death and survival

The first evidence for C/EBP $\delta$ 's ability to promote cell death came from the observation that ectopic C/EBP $\delta$  exacerbates the death of growth factor-deprived mammary epithelial cells [85]. C/EBP $\delta$  also promotes the cell death required for lumen formation when transformed mammary epithelial cells are cultured as 3D acini on matrigel [56]. Indeed, the loss of C/EBP $\delta$  delays cell death at the onset of the mammary gland involution that follows lactation [70]. Furthermore, in the context of this physiological cell death, C/EBP $\delta$  is induced by STAT3 and is necessary for the downregulation of cyclin D1, upregulation of p53 and IGFBP5, and proper regulation of several members of the Bcl family [70]. C/EBP $\delta$ 's ability to promote cell death in mammary epithelial cells suggests that it has potential as a tumor suppressor for breast cancer. In support of this notion, C/EBP $\delta$  expression is downregulated with breast cancer progression [108], and the loss of C/EBP $\delta$  increases tumor multiplicity in the MMTV-Neu mammary tumor model [34].

Interestingly, the transcription factor STAT3, which promotes cell death during mammary gland involution [109] is, on the other hand, frequently activated in breast cancer [110], and promotes metastasis in the MMTV-Neu mouse model, along with expression of *Cebpd* and an inflammatory and proangiogenic signature [111]. Indeed, despite the increased number of tumors in *Cebpd* KO mice, the loss of *Cebpd* results in a significantly reduced incidence of lung metastasis, which correlates with C/EBP $\delta$ 's role in supporting HIF-1 $\alpha$  expression and the consequent cell survival under hypoxia [34]. In addition, C/EBP $\delta$  can promote cell survival in response to DNA-damaging agents (see next section for more detail). Given that growth arrest is necessary for DNA repair and adaptation to hypoxia, it is conceivable that the growth arrest functions of C/EBP $\delta$  contribute to its promotion of survival under stress conditions.

In another example of C/EBP $\delta$ 's role in cell death, the  $\beta$ -diketone anti-cancer drug HMDB induces its expression, and C/EBP $\delta$  augments cervical tumor cell death and the activation of PPARG2 and

GADD153/CHOP (CHOP) transcription in response to HMDB [99]. On the other hand, C/EBP $\delta$  protects pancreatic  $\beta$  cells from cytokine-induced cell death by attenuating the expressions of GADD153/CHOP and STAT1 [112]. These are examples of the cell type- and context-dependent functions of C/EBP $\delta$ , as the same target gene(s) may be associated with opposite outcomes in different cell types.

Collectively, these reports indicate that C/EBP $\delta$  by itself is not a cell death inducer. Rather, C/EBP $\delta$  can promote cell death or survival, perhaps as a cell type- and context-dependent interpreter of extracellular signals.

### C/EBP $\delta$ and genome integrity

Several reports indicate a role for C/EBP $\delta$  in the DNA damage response and genomic integrity. C/EBP $\delta$  renders urothelial bladder carcinoma cell lines resistant to cisplatin, due to its role in expression of the Cu/Zn-superoxide dismutase SOD1, which scavenges reactive oxygen species [113]. The loss of C/EBP $\delta$  results in centrosome amplification and genomic instability in cultured mouse embryo fibroblasts (129S1 strain) [87]. This may be due in part to C/EBP $\delta$ 's role in promoting the degradation of cyclin D1 [89] and in the Fanconi Anemia (FA) replication-associated DNA repair pathway. C/EBP $\delta$  promotes the nuclear import of FANCD2, a critical protein for the FA pathway, by physically interacting with FANCD2 and the importin IPO4. This so-far unique non-transcriptional function of C/EBP $\delta$  may underlie its role in promoting cell survival in response to the DNA-damaging agent mitomycin C [41]. However, the loss of C/EBP $\delta$  is not sufficient to cause tumors in mice. Hence, the role of C/EBP $\delta$  in genomic stability may be compensated for *in vivo* and/or is only critical when cells are challenged by stress conditions such as *in vitro* or microenvironmental stressors, drugs, or mutations. On the other hand, C/EBP $\delta$  can also contribute to genomic instability by directly inducing the expression of Aurora C kinase, which leads to centrosome abnormalities in cervical carcinoma cells that have been treated with TNF $\alpha$  [114]. This last study highlights how C/EBP $\delta$  may switch functions specifically under inflammatory conditions.

### Role of C/EBP $\delta$ in inflammatory responses

One of the earliest discoveries about C/EBP $\delta$  was that its expression is induced by LPS from gram-negative bacteria and by pro-inflammatory cytokines in many cell types and tissues [1]. Studies of C/EBP $\delta$  in inflammatory signaling have focused on cells of the myeloid lineage [1]. Collectively, the re-

ports suggest a role for C/EBP $\delta$  in the activation of the innate immune response and in the generation of pro-inflammatory conditions. However, a few reports also point to possible functions of C/EBP $\delta$  in the inhibition of inflammation. In addition, other C/EBP family members play critical roles in the innate immune response and inflammation (e.g.[115,116,5]), and it remains to be determined how mutual regulation or distinct functions are played out in the overall control of the inflammatory response.

C/EBP $\delta$  amplifies LPS signaling in macrophages, supports the expression of many LPS-induced genes, and is necessary to clear persistent gram-negative bacterial infections [21]. Innate immune responses are largely mediated by toll-like receptors (TLRs), which act as a primary line of defense against pathogens, and activate the NF $\kappa$ B and IRF3 transcription program [117]. Mechanistically, C/EBP $\delta$  promotes LPS responses in part by inducing the expression of the LPS-receptor TLR4 and by inhibiting FBXW7 expression. In turn, FBXW7 $\alpha$  downregulates C/EBP $\delta$  expression and is an attenuator of inflammatory signaling [34, 57]. This negative feedback loop reveals an important role of the C/EBP $\delta$ /FBXW7 $\alpha$  pathway in inflammatory signaling and opens up new avenues for investigation of their functions in immune cells and inflammation-associated diseases.

C/EBP $\delta$  also binds directly and activates the promoters of many LPS-induced genes [21]. However, because C/EBP $\delta$  also induces expression of the LPS receptor subunit TLR4 [57], some of the reported defects in LPS responses due to *Cebpd*-null mutations or siRNA could also be due to reduced levels of the LPS receptor itself. For example, TLR4 reconstitution partially rescues the LPS-induced IL-6 expression in *Cebpd* null cells [57]. Interestingly, a low dose of LPS specifically induces C/EBP $\delta$  expression rather than NF $\kappa$ B [118], and the suppressor of cytokine signaling 3 (SOCS3) attenuates LPS-induced activation of C/EBP $\delta$  but not of NF $\kappa$ B [119]. Together, these findings suggest that C/EBP $\delta$  is critical in sensitizing cells to LPS and prepares them for activation of the innate immune response. Accordingly, C/EBP $\delta$  is necessary for lung infiltration by macrophages and to clear pulmonary infection by *Klebsiella pneumoniae* [75]. On the other hand, the loss of C/EBP $\delta$  protects against infection by *S. pneumoniae* [120]. While this result appears at odds with the aforementioned reports, this particular phenotype is explained by the role of C/EBP $\delta$  in lung epithelial expression of PAFR, the receptor used by *S. pneumoniae* to enter the organism [120]. Therefore, the relative resistance of *Cebpd*-null mutant mice to *S. pneumoniae* is unrelated to C/EBP $\delta$ 's role in the immune system.

Although inflammatory responses are essential for activation of the innate immune response and to enhance adaptive immunity, excessive inflammation is detrimental and can lead to autoimmune disorders and cancer (or sepsis in the case of bacterial triggers) [121]. Indeed, a *Cebpd* null mutation partly protects mice from sepsis, i.e. disseminated intravascular coagulation (DIC)-induced mortality in response to LPS [122]. Furthermore, C/EBP $\delta$  deficiency protects against LPS-induced lung injury [82]. In contrast, C/EBP $\delta$  deficiency does not protect against immune-complex-induced lung injury [83], although it participates in Fc $\gamma$  receptor-mediated activation of cultured macrophages [79]. These data indicate that the role of C/EBP $\delta$  in inflammation depends in part on the inflammatory stimulant. Because most studies so far have used LPS, C/EBP $\delta$ 's role in the response to other specific triggers of the innate immune response still needs to be determined. Interestingly, a *Cebpd* null mutation has profound effects on the expression of several TLR genes in peritoneal macrophages [57], raising the possibility that C/EBP $\delta$  has a much broader role in the innate immune response beyond LPS.

So-called sterile inflammation in the absence of foreign antigens also contributes to pathological conditions. *CEBPD* expression is induced by inflammatory cytokines, including IL-6, and in turn C/EBP $\delta$  directly activates IL-6 gene expression [123], providing the potential for an autocrine pro-inflammatory feedback loop. C/EBP $\delta$  expression in astrocytes is implicated in the pathology of Alzheimer disease through the activation of inflammatory cytokines and pentraxin-3 (PTX3), a secreted molecule shown to inhibit the phagocytosis of damaged neurons by macrophages [124, 125]. In CNS dendritic cells, C/EBP $\delta$  suppresses the expression of IL-10, consequently altering the ratio of T helper17 cells (Th<sub>17</sub>) and T regulatory cells (T<sub>reg</sub>), and thereby contributing to the severity of experimental autoimmune encephalomyelitis [126]. C/EBP $\delta$  is also implicated in the promotion of amyotrophic lateral sclerosis, because of its expression in the activated microglia of patients and in a mouse model of this disease, and because it promotes the expression of inflammatory cytokines in the brain in response to systemic LPS [127]. Indeed, the anti-inflammatory flavanoid chrysin inhibits microglial C/EBP $\delta$  expression and the concomitant neural cell death in culture [128].

In another inflammatory pathology, C/EBP $\delta$  promotes the pathogenesis of collagen-induced rheumatoid arthritis in mice, most likely by contributing to the expression of chemokines and cytokines in macrophages that promote the formation of endothelial cell tubes and the migration and proliferation

of synoviocytes [129]. In addition, C/EBP $\delta$  mediates the IL-1 $\beta$ -induced expression of secreted type IIA phospholipase A(2) in chondrocytes, which can also contribute to rheumatoid arthritis [130]. Taken together, these reports strongly support a role for C/EBP $\delta$  in the pathology of chronic inflammation and autoimmune disorders.

A few reports point to additional anti-inflammatory functions for C/EBP $\delta$ . For example, C/EBP $\delta$  expression can be induced by glucocorticoids and PGE<sub>2</sub>, which are potent anti-inflammatory and immunosuppressant agents [54, 131, 132]. Most strikingly, C/EBP $\delta$  attenuates the IL-1 $\beta$ +IFN- $\gamma$ -induced chemokine production in rat insulinoma cells by promoting the expression of IRF-1 [112]. The role of C/EBP $\delta$  in supporting or inhibiting inflammatory signaling may depend on the context or cell type. Indeed, C/EBP $\delta$  activates the expression of anti-inflammatory IL-10 in response to LPS or PGE<sub>2</sub> in macrophages [54, 133]. On the other hand, a *Cebpd* null mutation leads to increased IL-10 expression in dendritic cells, which is associated with the progression of CNS autoimmune inflammatory disease [126]. Elevated IL-10 mRNA levels have also been observed in *Cebpd* null mammary tumor tissue [57]; however, it remains to be determined if this is due to a direct or indirect role of C/EBP $\delta$ .

Negative feedback regulation of C/EBP $\delta$  expression appears to play an important role in the resolution of inflammation and can be achieved through ATF-3 [21], Myc, and the Myc-interacting protein Miz1 [134, 135]. It will be very interesting to learn if and how C/EBP $\delta$ , ATF3, and the Myc/Miz1 complex engage in mutual regulatory feedback loops to modulate the magnitude and duration of inflammatory signaling.

Interestingly, C/EBP $\delta$  can activate an indirect negative feedback mechanism by inducing the expression of the peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ), a member of the nuclear receptor superfamily, in VSMCs. PPAR $\gamma$  plays a key role in the inhibition of inflammation, in part by inactivating the STAT3 transcription factor, thereby downregulating *CEBPD* expression [136]. PPAR $\gamma$  agonists are currently in clinical trials for the treatment of many inflammatory disorders [137]. Thus, by inducing PPAR $\gamma$  expression, C/EBP $\delta$  contributes to the attenuation of inflammatory responses in the early stages of atherosclerosis [136]. On the other hand, *sumoylated* C/EBP $\delta$  is reported to inhibit PPAR $\gamma$  gene transcription in hepatocytes [36]. Therefore, the regulation of C/EBP $\delta$ 's sumoylation may provide a mechanism for increasing the inflammatory response. The potential for another feedback loop was raised in a report on the role of IKKi/IKK $\epsilon$  (IKBKE) in the LPS induction of

C/EBP $\delta$  expression and activity, in which C/EBP $\delta$  was found to bind to the *IKBKE* promoter [61]. However, it is not known if *IKBKE* expression is reduced or activated by C/EBP $\delta$ .

As mentioned earlier, C/EBP $\delta$  also inhibits its own suppressor. C/EBP $\delta$  protein is targeted for ubiquitination and degradation by FBXW7 $\alpha$ , and *FBXW7a* gene expression is in turn inhibited by C/EBP $\delta$ . This negative feedback loop depends on GSK-3 $\beta$ 's phosphorylation of C/EBP $\delta$ , which is inhibited by LPS. Therefore, LPS leads to the stabilization of C/EBP $\delta$  [57]. Interestingly, GSK3 $\beta$  is mostly known as a driver of inflammation, and its inhibitors are under investigation as therapeutics for a variety of diseases [138]. The discovery that GSK-3 $\beta$  can promote the degradation of C/EBP $\delta$  therefore opens up new avenues for investigation into the cross-talk between signaling pathways and the regulation of the innate immune response and inflammation.

### C/EBP $\delta$ expression in human cancers

In accordance with the diversity of C/EBP $\delta$  functions described above, analyses of C/EBP $\delta$  expression in human cancers paint a similarly complex picture, indicating that it functions both as a tumor suppressor and tumor promoter. In breast cancer, *CEBPD* mRNA is downregulated with malignant progression, i.e. it is significantly reduced or undetectable in invasive/metastatic disease [108, 139, 140], and *CEBPD* is part of a 70-gene signature that correlates with longer survival of breast cancer patients [141]. These data suggest that C/EBP $\delta$  has a tumor-suppressor function. On the other hand, C/EBP $\delta$  protein expression is positively correlated with cyclin D1 and cyclin E in human breast cancer tissue [72], in contrast to several reports showing that C/EBP $\delta$  downregulates these cyclins in cultured cell models [85, 89, 91, 93]. While this study [72] did not analyze the correlation of C/EBP $\delta$  expression with survival/prognosis, proliferation markers are usually correlated with poor prognosis. Thus, it will be very interesting to follow up on this initial report with a more extensive analysis of C/EBP $\delta$  protein expression in breast cancer, especially given that findings from a mouse model suggest that C/EBP $\delta$  has a dual activity as tumor suppressor and a promoter of metastasis [34].

In support of a tumor suppressor function, C/EBP $\delta$  protein expression correlates with longer disease-free survival in meningioma patients [73]. *CEBPD* gene methylation was identified in breast, liver, and cervical carcinomas [142, 143]. A recent report suggests that metastatic lesions in breast cancer patients exhibit a higher incidence of methylation

within a CpG island of *CEBPD*, which was associated with increased risk of relapse [144]. Consistent with the observation that C/EBP $\delta$  promotes myeloid differentiation in cell culture models (see above), the *CEBPD* promoter was found to be silenced in 35% of 260 human acute myeloid leukemia cases [8] and its expression is downregulated in the blast crisis phases of CML [100]. Interestingly, in the U937 leukemia cell line, eicosapentaenoic acid causes the demethylation of a specific CpG in the *CEBPD* promoter, increased C/EBP $\delta$  expression, and cell differentiation [145]. Furthermore, the *CEBPD* gene resides within a common fragile site that is frequently lost in certain cancer cell lines. However, it is not known if this finding has any functional bearing on the behavior of tumor cells or is a byproduct of *CEBPD*'s vicinity to the *KIAA0146* gene [146]. While these data are consistent with a tumor-suppressor function of C/EBP $\delta$ , mutations in *CEBPD* are not common in breast cancer [147]. In addition, analyses of the *CEBPD* gene sequence in leukemias, lymphomas, and a variety of solid tumors indicate that mutations of *CEBPD* may not contribute to the development of neoplastic disease [148]. Interestingly, single nucleotide polymorphisms in the *CEBPD* gene are infrequent, perhaps indicative of a stringent need for gene conservation, and none has so far been associated with disease(s) [149, 150].

As in breast cancer, *CEBPD* mRNA is downregulated in human and rat prolactinomas, and C/EBP $\delta$  inhibits prolactinoma cell proliferation in culture through the inhibition of prolactin expression [18, 151]. In contrast, *CEBPD* mRNA levels are upregulated in mesothelioma [152], gliomas [7], and in Anaplastic Large Cell Lymphoma but not in Hodgkin's Lymphoma [153]. However, correlation studies of expression and cancer biology will be more informative if performed at the protein level, because the C/EBP $\delta$  level can be regulated by its protein stability [56, 57], and the *CEBPD* mRNA expression level may not therefore correlate with the protein level and hence function. Furthermore, analysis in the context of tissue histology will be necessary in light of C/EBP $\delta$ 's potential expression in tumor-infiltrating host cells.

A strong case for C/EBP $\delta$  as a tumor promoter can be made in brain cancer. *CEBPD* mRNA is overexpressed in mesenchymal glioblastoma cells and associated with poor prognosis [7, 154]. C/EBP $\delta$  protein expression is detected near necrotic glioblastoma tissue [7]. Based on the observation that C/EBP $\delta$  promotes HIF-1 $\alpha$  expression and survival under hypoxic conditions [34], we speculate that this function is responsible for the association of C/EBP $\delta$  expression with poor prognosis in glioblastoma (see also section below). Furthermore, C/EBP $\delta$  inhibits the

expression of FBXW7 $\alpha$  in glioblastoma cell lines [34]. FBXW7 $\alpha$  is a substrate-binding subunit of the SCF E3 ubiquitin ligase complex and targets many oncoproteins for degradation, including mTOR, NOTCH, c-myc, and cyclin E [59] and is a *bona fide* tumor suppressor in glioblastoma [155]. Therefore, we suggest that C/EBP $\delta$ 's inhibition of FBXW7 $\alpha$  contributes to glioblastoma progression.

Taken together, expression studies in human neoplasias suggest that C/EBP $\delta$  can be associated with and contribute to either a better or worse prognosis, depending on the tumor type or cell of origin. Given the role of C/EBP $\delta$  in inflammation and the cross-talk between tumor cells and the immune system, care must be taken to determine if the expression of C/EBP $\delta$  is associated with a cell differentiation state, hypoxic or inflammatory induction, or is a result of immune cell infiltration.

### Role of C/EBP $\delta$ in cancer: a special case for hypoxia and inflammation?

Accumulating evidence supports a role for hypoxia and inflammation in the progression of tumors [156, 157]. As outlined above, C/EBP $\delta$  expression is induced by inflammatory effectors and hypoxia, and it promotes pro-inflammatory signaling and adaptation to hypoxia. Thus, C/EBP $\delta$  expression and functions are strongly associated with conditions that correlate with cancer progression, which is in contrast to its initial designation as a tumor suppressor. Mechanistically, one C/EBP $\delta$  pathway stands out as highly relevant for both conditions. Inhibition of the tumor suppressor FBXW7 $\alpha$  by C/EBP $\delta$  leads to the upregulation of many oncoproteins and indirectly to the upregulation of HIF-1 $\alpha$  [34, 57], which has a prominent role in hypoxia adaptation, inflammation, and cancer [59, 158]. Consistent with this notion, MMTV-Neu-induced mammary tumors arising in *Cebpd* null mice are less metastatic than those arising in control mice [34], and the growth of experimental metastases of a lung cancer cell line is impaired in *Cebpd* null host mice [48]. Furthermore, C/EBP $\delta$  is associated with a mesenchymal phenotype and poor prognosis in glioblastoma [7, 154].

The association of C/EBP $\delta$  with hypoxia and inflammation raises additional questions. For example, although HIF-1 $\alpha$  has many pro-oncogenic functions, it too can act as a tumor suppressor or promote therapeutic responses [159]. Indeed, HIF-1 $\alpha$  can promote cell survival or cell death depending on the degree of hypoxia [160]. However, it is unclear if C/EBP $\delta$  supports HIF-1 $\alpha$  expression and function under all of these conditions. Furthermore, it remains to be determined how HIF-1-independent functions of

C/EBP $\delta$  affect cells under hypoxic conditions and whether HIF-1 induces *CEBPD* expression directly.

Monocyte and dendritic cell infiltrations are seen in the hypoxic regions of solid tumors, particularly associated with necrotic tissue, and HIF-1 $\alpha$  is required for the recruitment of myeloid cells [161]. Does C/EBP $\delta$  support the recruitment of monocytes to the tumors and/or the function of tumor-associated macrophages (TAMs)? Altered cytokine/chemokine expression patterns in *Cebpd* null mammary tumors suggest that the answer is yes [57]. The immune system's role is to eliminate tumor cells, and subversion of the immune system by the tumor leads to escape from immune surveillance and the promotion of chronic inflammatory signaling. Interestingly, C/EBP $\delta$  promotes the differentiation of bone-marrow macrophages to the classical M1 type, while *Cebpd* silencing promotes the alternatively activated M2-type, which is considered immunosuppressive [55]. This finding suggests a potential tumor-suppressor function of C/EBP $\delta$  in the immune system. Indeed, it is conceivable that C/EBP $\delta$  functions in the innate immune response may contribute to the reduced tumor burden of *Cebpd* null mice in the mammary tumor model [34]. On the other hand, a very recent study indicates that C/EBP $\delta$  expression in TAMs correlates by several measures with the malignant progression of nasopharyngeal carcinoma [54]. Thus, an important question to resolve is the role of C/EBP $\delta$  in tumor cells as opposed to in the tumor microenvironment along tumor progression. It needs to be determined what types of immune cells express C/EBP $\delta$  *in vivo* and how C/EBP $\delta$ 's functions in these cells affect the metastatic phenotype. The dual role of C/EBP $\delta$  is reminiscent of the "TGF- $\beta$  paradox" [11, 12]. While TGF- $\beta$  has been shown to inhibit C/EBP $\delta$  expression and activity in VSMCs and pre-adipocytes respectively [40, 162] an interesting open question is the extent of cross-talk between TGF- $\beta$  and C/EBP $\delta$  signaling in tumor and immune cells. A conditional null allele of C/EBP $\delta$ , which is being developed in our laboratory, will be useful for addressing some of the above questions in mouse models.

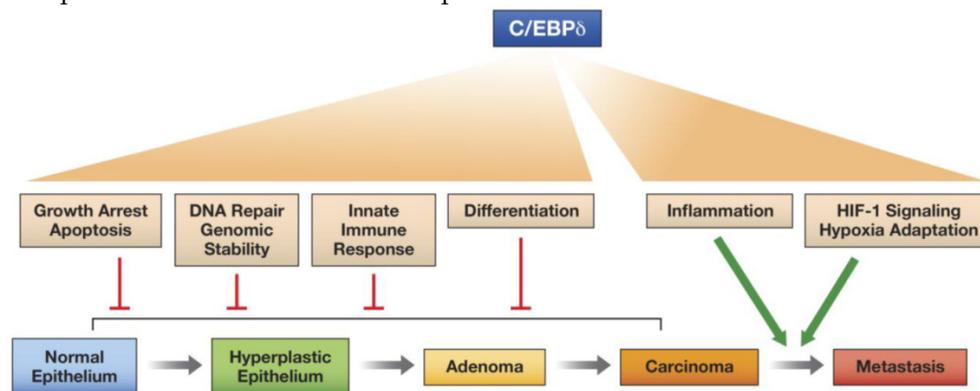
### Summary and Perspectives

In this review we have highlighted the versatile nature of the C/EBP $\delta$  protein, a transcription factor that can activate or repress target genes and promotes many different, and sometimes contrasting, cellular functions, depending on context, that can affect intracellular functions and/or the microenvironment. C/EBP $\delta$  even exhibits at least one documented non-transcriptional function. We propose that C/EBP $\delta$  has potential as a tumor suppressor when it inhibits proliferation, promotes differentiation, con-

tributes to genomic integrity, and supports the innate immune response. On the other hand, C/EBP $\delta$  promotes tumor progression through its functions in hypoxia adaptation and pro-inflammatory signaling, which may also operate in the tumor parenchyma and the tumor microenvironment (Figure 2).

Far too little is known about physiologically relevant C/EBP $\delta$  protein modifications or how spe-

cific protein interactions are regulated and affect the regulation of C/EBP $\delta$  target genes and C/EBP $\delta$ 's cell-type-specific activities. Because C/EBP $\delta$  is linked to so many cellular functions, insights into its roles and regulation will lead to a better understanding of the complexity and diversity of cellular processes in the normal development and progression of diseases.



**Fig 2. Model for the differential effects of C/EBP $\delta$  on epithelial tumor progression.** C/EBP $\delta$  may function as a tumor suppressor at the early stages of tumor development, because of its role in promoting growth arrest, cell death, differentiation, DNA repair, genomic stability, and the innate immune response. However, by supporting HIF-1 activity and hypoxia adaptation and by amplifying inflammatory signaling, C/EBP $\delta$  promotes tumor progression.

## Acknowledgements

The authors are supported by the Intramural Research Program of the NIH, National Cancer Institute. We are grateful to Drs. Peter Johnson, Howard Young, and Ju-Ming Wang for helpful discussion and critical comments on the manuscript, and Allen Kane for preparation of the figures. We apologize for not citing all the publications in the field and welcome feedback.

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## Competing Interests

The authors have declared that no conflict of interest exists.

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