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The Wnt-target gene Dlk-1 is regulated by the Prmt5-associated factor Copr5 during adipogenic conversion

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ABSTRACT
Protein arginine methyl transferase 5 (Prmt5) regulates various differentiation processes, including adipogenesis. Here, we investigated adipogenic conversion in cells and mice in which Copr5, a Prmt5- and histone-binding protein, was genetically invalidated. Compared to control littermates, the retroperitoneal white adipose tissue (WAT) of Copr5 KO mice was slightly but significantly reduced between 8 and 16 week/old and contained fewer and larger adipocytes. Moreover, the adipogenic conversion of Copr5 KO embryoid bodies (EB) and of primary embryo fibroblasts (Mefs) was markedly delayed. Differential transcriptomic analysis identified Copr5 as a negative regulator of the Dlk-1 gene, a Wnt target gene involved in the control of adipocyte progenitors cell fate. Dlk-1 expression was upregulated in Copr5 KO Mefs and the Vascular Stromal Fraction (VSF) of Copr5 KO WAT. Chromatin immunoprecipitation (ChIP) show that the ablation of Copr5 has impaired both the recruitment of Prmt5 and β-catenin at the Dlk-1 promoter. Overall, our data suggest that Copr5 is involved in the transcriptional control exerted by the Wnt pathway on early steps of adipogenesis.

KEY WORDS: β-catenin, Copr5, Dlk-1, Prmt5, Adipocyte, Differentiation

INTRODUCTION
Adipose tissue has various regulatory functions in the metabolism of animals and acts both as a fat reservoir and an endocrine/paracrine/autocrine organ that can expand throughout the entire lifespan. This functional plasticity can lead to pre-adipocyte hyperplasia and adipocyte hypertrophy. Fat tissue includes many different cellular components, including preadipocytes, multipotent stem cells (MSC) and mature adipocytes (Zeve et al., 2009).

Adipogenesis is a multi-step process during which the increase in adipocyte number is triggered by various extra- and intra-cellular signalling factors that induce MSC conversion into preadipocytes (Tang and Lane, 2012). This commitment is restricted to the adipocyte lineage upon activation of a transcriptional programme in which key factors of adipocyte differentiation like C/EBPz and Pparγ are induced (MacDougald and Lane, 1995; Rosen and MacDougald, 2006; Tontonoz and Spiegelman, 2008). Among sensors of external signals to trigger adipocyte differentiation during embryonic development and adult life, the Wnt signalling pathway is crucial for progenitor fate determination and acts through Dlk-1 that regulates negatively preadipocyte proliferation (Moon et al., 2002; Mortensen et al., 2012; Smas and Sul, 1993).

In association with protein complexes involved in different phases of transcription, Protein arginine methyl transferase 5 (Prmt5) was implicated in myogenic, adipogenic and glial cell differentiation (Dacwag et al., 2009; Huang et al., 2011; LeBlanc et al., 2012; Paul et al., 2012). Consistently with this biological function, we reported previously that the depletion of the Prmt5 and histone-associated protein Copr5 delays the myogenic conversion (Paul et al., 2012), suggesting that Copr5 elicits a fine tuning of Prmt5 functions related to cell differentiation.

In this work, we generated mice in which Copr5 was genetically invalidated and show that adipogenic conversion was delayed in vitro both in EBs and Mefs derived from these mice compared to control cells. In addition, the retroperitoneal WAT of Copr5 KO (KO) mice was slightly reduced and contained larger adipocytes compared to control mice. Finally, we show that the expression of Dlk-1 was upregulated in KO cells and coincides with an altered recruitment of Prmt5 and β-catenin to the Dlk-1 promoter. Altogether, our data highlight unsuspected functions of Copr5 in the modulation of adipogenic differentiation, notably through an impact on the Wnt/β-catenin-dependent regulation of the Dlk-1 promoter.

RESULTS AND DISCUSSION
Adipogenesis is impaired in Copr5 KO cells
We generated a mouse model in which the Copr5 gene was genetically invalidated by homologous recombination (supplementary material Fig. S1). In contrast to Prmt5 loss of function, which is early embryonic-lethal due to loss of pluripotent cells (Tee et al., 2010), Copr5 KO mice were viable and ES cells could be derived from KO blastocysts, indicating that the Copr5-independent functions of Prmt5 are not essential for mouse development. However, when tested for their capacity to differentiate in vitro into adipocytes (Dani et al., 1997), lipid droplets were observed mostly in WT EBs cultures at D21 (Fig. 1A). Moreover, the mRNA level of Myf5, which was used as a read-out of differentiation, confirmed that mesodermic lineage differentiation was already delayed at D4 in KO compared to WT EBs (Fig. 1B). O Red Oil staining and mRNA analysis showed that adipogenic conversion was also very ineffective in KO compared to WT Mefs (Fig. 1C,D), as well as in Copr5 shRNA-treated F-442A preadipocyte cell line (supplementary material Fig. S2D,E).
Altogether, these data indicate that Copr5 deficiency impacted on very early and irreversible events required for the adipogenic conversion of Mefs.

**Copr5 controls the expression of Dlk-1 gene, a key regulator of preadipocyte differentiation**

To unravel the molecular mechanisms that could explain the poor capacity of KO Mefs to undergo an adipogenic conversion, we compared their transcriptome profile with that of WT Mefs (supplementary material Table S1). Notably, among the 538 genes that were significantly deregulated (Zr>2;Zpval>0.05) in KO cells, 34 were bona fide Wnt/β-catenin target genes (p=4.67x10^-12, Fisher’s test) (supplementary material Fig. S3A–C). Biochemical fractionation showed that KO Mefs contained higher amounts of the activated form of β-catenin in their nucleus than WT cells (supplementary material Fig. S3D), a difference that was lessened upon treatment with either LiCl or C59, two chemicals known to activate and inhibit the Wnt pathway, respectively (supplementary material Fig. S3E). Consistently, reporter assays confirmed that TCF/β-catenin transcriptional activity was increased in KO cells (supplementary material Fig. S3E). Within this list, we noticed the presence of Dlk-1, a gene encoding a key regulator of adipose tissue homeostasis *in vivo* whose expression in WAT is linked to inhibition of adipocyte differentiation (Moon et al., 2002; Mortensen et al., 2012; Smas and Sul, 1993). Interestingly, *Dlk-1* is one of the few non-conventional target genes of the Wnt pathway that were reported to be directly repressed by the TCF/β-catenin complex (Blauwkamp et al., 2008; Weng et al., 2009). Analysis of *Dlk-1* expression confirmed its sensitivity to LiCl in WT Mefs and its upregulation in KO Mefs (Fig. 2A–C), suggesting that this gene was derepressed in KO cells, despite their high levels of activated β-catenin. Based on our previous reports showing that Copr5/Prmt5 complex could be involved in transcriptional repression (Lacroix et al., 2008), we hypothesised it could be involved in the repression of the *Dlk1* promoter. Consistently, ChIP performed in Mefs during the early phase of their adipogenic conversion showed that Prmt5 was present on the *Dlk1* promoter in WT but not KO Mefs (Fig. 2D). Similarly, the association of β-catenin on the two TCF binding sites (TCFbs 1 and 2) present on this promoter was significantly reduced in KO Mefs (Fig. 2E), suggesting that Copr5/Prmt5 is required for β-catenin recruitment and TCF-mediated transcriptional repression of *Dlk-1*. Of note, we failed to detect a direct protein-protein interaction between Copr5/Prmt5 and β-catenin in vitro (data not shown). Interestingly, we found that the recruitment of Brg-1, a chromatin remodeler that can be recruited by β-catenin to TCF target gene promoters and able to interact with Prmt5 (Curtis and Griffin, 2012; de la Serna et al., 2001; Griffin et al., 2011), decreased slightly in KO compared to WT cells (supplementary material Fig. S4). To which extent proteins that are able to antagonise β-catenin/TCF activity might be responsible of this reduced binding of β-catenin at the *Dlk-1* promoter in KO cells still remains. We next assessed whether a shRNA-mediated depletion of *Dlk-1* could restore the capacity of these cells to differentiate. Because they differentiated poorly once infected with shRNAs, we used Copr5-depleted F442A cells. Although a reduction of *Dlk-1* level was obtained in these cells, this...
repressor complex on this promoter.

Cathepsin binding and formation of a functional TCF-associated Dlk-1 recruitment on the Dlk-1 gene expression in Mefs and suggest that Copr5/Prmt5 Dlk-1 exclusively on cellularity in containing VSF of the WAT and modification of the adipocyte cellularity observed in KO WAT is a consequence of Dlk-1 upregulation, leading consequently to a low pool of precursor cells that is able to differentiate into adipocytes. This impaired adipocyte differentiation resulted, at least in part, from a reduced recruitment of Prmt5, Brg1 and β-catenin to the Dlk-1 promoter in KO mice.

Altogether, our data suggest a model depicted in Fig. 4 in which modification of the adipocyte cellularity observed in KO WAT is a consequence of Dlk-1 upregulation, leading consequently to a low pool of precursor cells that is able to differentiate into adipocytes. This impaired adipocyte differentiation resulted, at least in part, from a reduced recruitment of Prmt5, Brg1 and β-catenin to the Dlk-1 promoter in KO mice.

Further studies are now required to understand whether Prmt5/Copr5 complex participates in the transcriptional regulation of other β-catenin-regulated genes that are deregulated in KO Mefs. It will be also interesting to explore whether this complex controls Dlk-1 expression in the other few adult tissue/glands/neurons that maintain an expression of Dlk-1, and whether a deregulation of its expression generated subtle yet unidentified phenotypes in these organs.

Upregulation of Dlk-1 expression in progenitor cell containing VSF of the WAT and modification of the adipocyte cellularity in Copr5 KO mice

 Knockout and WT male mice were indistinguishable with a similar mean body weight (Fig. 3A, left panel). Histological analysis did not reveal significant morphological changes (C.P., E.F., data not shown), excepted in retroperitoneal adipose tissue. A more in depth analysis revealed that its mass was moderately, but reproducibly decreased in KO compared to controls at 16 weeks of age (Fig. 3A, right panel) and that it contained fewer adipocytes but of larger size than WT tissue (Fig. 3B). Of note, glucose and insulin tolerance tests were similar in both types of mice, ruling out major alteration of the glucose and insulin-dependent metabolic axis (data not shown). In agreement with our findings in ES cells and Mefs, and consistent with a reduction in the adipocyte number, a phenotype also encountered in transgenic mice for Dlk-1 (Lee et al., 2003), the mRNA levels of aP2, Lpl, C/EBPα, C/EBPβ and Pparγ were downregulated, whereas that of Dlk-1 was upregulated in KO WAT, supporting a role of Copr5 in controlling adipogenesis in vivo (Fig. 3C). Hence, we hypothesised that the large adipocytes detected in KO mice could reflect an adaptive response to a reduced proliferation/differentiation of KO preadipocytes. Consistently, the proliferation index of the VSF, a main source of progenitor cells, was lower in KO compared to WT mice (Fig. 3D) and associated with a strong upregulation of Dlk-1 expression, whereas no difference was noted in mature adipocytes (Ad), as expected (Fig. 3E). In addition, we found that the Dlk-1 membrane-bound isoform (Dlk-1) which exerts a negative effect on preadipocyte proliferation (Mortensen et al., 2012), was increased in WAT (Fig. 3F).

Altogether, our data suggest a model depicted in Fig. 4 in which modification of the adipocyte cellularity observed in KO WAT is a consequence of Dlk-1 upregulation, leading consequently to a low pool of precursor cells that is able to differentiate into adipocytes. This impaired adipocyte differentiation resulted, at least in part, from a reduced recruitment of Prmt5, Brg1 and β-catenin to the Dlk-1 promoter in KO mice.

Further studies are now required to understand whether Prmt5/Copr5 complex participates in the transcriptional regulation of other β-catenin-regulated genes that are deregulated in KO Mefs. It will be also interesting to explore whether this complex controls Dlk-1 expression in the other few adult tissue/glands/neurons that maintain an expression of Dlk-1, and whether a deregulation of its expression generated subtle and yet unidentified phenotypes in these organs.
MATERIALS AND METHODS

Cell culture conditions
Adipogenic differentiation was induced in post-confluent cells upon addition of a differentiation cocktail (50 nM insulin, 0.5 mM IBMX, 1 mM dexamethasone and 10^{-6} M rosiglitazone) to the medium. ES cells and EBs were cultivated as described previously (Dani et al., 1997).

Mice and animal care
Animal experiments were approved by the Ethics Committee of the Languedoc-Roussillon Region (France).

Vascular stromal fraction (VSF) isolation
Adipose tissue was dissected, washed in PBS with a 2% penicillin/streptomycin/gentamycin mixture, minced, and incubated in DMEM supplemented with 10 mg/ml BSA, 0.35% type II collagenase (SIGMA) at 37°C with shaking for 30 min. Cell suspensions were obtained after filtration through 100 μm cell strainers, centrifuged. The remaining pellet was resuspended, filtered through 40 μm cell strainers and centrifuged to recover the VSF.

Flow cytometry
In vivo labelling was performed by intraperitoneal injection of either BrdU at a concentration of 50 μg BrdU/g body weight or PBS included as negative controls in 7-week-old animals that were sacrificed seven days later for VSF isolation. VSF was processed using the BrdU FITC kit, as recommended by BD Pharmingen.

Determination of the adipocyte size
Sections of paraffin-embedded adipose tissue were stained with haematoxylin/eosin. Quantification was performed from images within a 500 μm measurement frame using ImageJ. Three independent measurements were performed in both WT and KO mice (n=3).

RNA isolation, cDNA synthesis and RT-qPCR amplification
RNA isolation and RT-qPCR were performed as described (Paul et al., 2012). The sequence of the oligonucleotides is listed in supplementary material Table S2.
Chromatin immunoprecipitation

Anti-Prmt5 and -β-catenin (S33/37/41) antibodies (Euromedex and Cell Signaling, respectively) were used for ChIP, as described (Paul et al., 2012). Sequence of the oligonucleotides is listed in supplementary material Table S2.

Western blot

Anti-Prmt5 (Millipore), -β-catenin (Cell Signaling), Histone H3 (Millipore), -Dlk-1 (Abcam) and -Tubulin antibodies were used.

Microarray analysis

Microarray analysis was performed using total RNA isolated from either Copr5 KO or WT Mefs from male embryos (n=3), hybridised onto a GeneChips® Mouse Gene 2.0 ST Array and analysed for differentially expressed genes (KFB, Germany) that were considered significant when the Z ratio and the adjusted Zp value was >2 and 0.05, respectively.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

CP and EF performed the experiments. EF conceived and designed the experiments. All participated in data analysis. EF and CS wrote the manuscript.

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