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To cite this version:
Conception Paul, Claude Sardet, Eric Fabbrizio. The Wnt-target gene Dlk-1 is regulated by the Prmt5-associated factor Copr5 during adipogenic conversion. Biology Open, Royal Society, 2015, 4 (3), pp.312-316. 10.1242/bio.201411247. hal-02277973

HAL Id: hal-02277973
https://hal.umontpellier.fr/hal-02277973
Submitted on 4 Sep 2019

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RESEARCH ARTICLE

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

Conception Paul1,2, Claude Sardet1,2,3,* and Eric Fabbrizio1,2,3,*

ABSTRACT

Protein arginine methyltransferase 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 weeks 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 intracellular 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/EBPα 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 differentiation (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 had impacted on 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 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 ($Z_r > 2; Z_{pval} > 0.05$) in KO cells, 34 were bona fide Wnt/β-catenin target genes ($p = 4.67 \times 10^{-12}$, Fisher’s test) (supplementary material Fig. S2A–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. S3D). 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 (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), confirming that the recruitment of Brg-1, a chromatin remodeler that can interact with Prmt5 (Curtis and Griffin, 2012; de la Serna et al., 2011) and is known to recruit 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 Dlk1 promoter in KO cells still remains. We next assessed whether a shRNA-mediated depletion of Dlk1 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

![Fig. 1. Adipogenic conversion is delayed in Copr5 KO cells.](image-url)
repressor complex on this promoter. catenin binding and formation of a functional TCF-associated recruitment on the Dlk-1 gene expression in Mefs and suggest that Copr5/Prmt5 exclusively on Dlk-1 cellularity in containing VSF of the WAT and modification of the adipocyte Red Oil staining at D6 of sh 314 Dlk-1 mice for the adipocyte number, a phenotype also encountered in transgenic findings in ES cells and Mefs, and consistent with a reduction in dependent metabolic axis (data not shown). In agreement with our mice, ruling out major alteration of the glucose and insulin-glucose and insulin tolerance tests were similar in both types of adipocytes but of larger size than WT tissue (Fig. 3B). Of note, weeks of age (Fig. 3A, right panel) and that it contained fewer but reproducibly decreased in KO compared to controls at 16 A more in depth analysis revealed that its mass was moderately, E.F., data not shown), excepted in retroperitoneal adipose tissue. analysis did not reveal significant morphological changes (C.P., similar mean body weight (Fig. 3A, left panel). Histological Knockout and WT male mice were indistinguishable with a membrane-bound isoform (Dlk-1) which exerts a negative effect from a reduced recruitment of Prmt5, Brg1 and 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 Copr5 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.

Fig. 2. Dlk-1 upregulation in Copr5 KO Mefs is related to impaired recruitment of Prmt5 to the Dlk-1 promoter. (A) Expression of indicated mRNAs in Mefs was monitored by RT-qPCR after treatment of the cells with LiCl (10 mM) for 24 h. Results are expressed in arbitrary units (a.u.). (B) Expression of Dlk-1 mRNA in WT and KO Mefs was monitored by RT-qPCR at D0 and D7 of differentiation. Normalisation and expression was done as in Fig. 1D. (C) Western Blot detection of WT and KO Mef whole cell extracts treated as in (A) using an anti-Dlk-1 antibody is shown. (D) Prmt5 recruitment to A and B regions of the Dlk-1 promoter in WT and KO Mefs was analysed by ChIP at D0 and D7. Values are expressed as the percentage of immunoprecipitated (ip) chromatin relative to input and are the mean±s.e.m. of triplicates. No antibody (noAb) was used as negative control. (E) Immunoprecipitation was performed as in (D) to assess the recruitment of activated β-catenin (β-cat) to three regions of the Dlk-1 promoter: two of them encompass the TCF binding sites 1 and 2 (TCFbs1 and TCFbs2), the third one is B, as in (D). (F) Expression of Dlk-1 mRNA in shCopr5 F442A-treated cells was monitored by RT-qPCR upon inactivation of Dlk-1 using shRNA (shDlk1) encoding retroviral particles. A scramble shRNA (shScr) was used as control. Normalisation and expression were performed as in (B). (G) O Red Oil staining at D6 of shCopr5 F442A-treated cells infected as in (F). *: non specific band.
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/gentamicin 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 Pharamingen.

Determination of the adipocyte size

Sections of paraffin-embedded adipose tissue were stained with haematoxylin/eosin. Quantification was performed from images within a 500×500 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 Cop5 KO or WT Mefs from male embryos (n=3), hybridised onto a GeneChip® 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.

Acknowledgements
We would like to thank specially C. Chavey for insulin tolerance tests, C. Pescia-Begon for ES cell isolation and the RHEM facility for tissue sections. We are grateful to all members of CS laboratory. This work was realised with the institutional support of the French CNRS.

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.

Funding
This work was supported by grants from the Ligue Contre le Cancer [to C.S., 2012–2014] and the Association pour la Recherche contre le Cancer (grant 493 to E.F.).

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