BBA - Gene Regulatory Mechanisms 1860 (2017) 1079–1093

Contents lists available at ScienceDirect

BBA - Gene Regulatory Mechanisms

journal homepage: www.elsevier.com/locate/bbagrm

The histone lysine methyltransferase Ezh2 is required for maintenance of the intestine integrity and for caudal fin regeneration in zebrafish

Barbara Dupret[a,1], Pamela Völkel[a,b,1], Constance Vennin[a,c], Robert-Alain Toillon[a], Xuefen Le Bourhis[a], Pierre-Olivier Angrand[a,*]

[a] Cell Plasticity & Cancer, Inserm U908/University of Lille, Lille, France
[b] CNRS, Lille, France
[c] SIRIC ONCOLille, Lille, France

ARTICLE INFO

Keywords:
Zebrafish - TALEN - Polycomb repression
Ezh2
GSK126

ABSTRACT

The histone lysine methyltransferase EZH2, as part of the Polycomb Repressive Complex 2 (PRC2), mediates H3K27me3 methylation which is involved in gene expression program repression. Through its action, EZH2 controls cell-fate decisions during the development and the differentiation processes. Here, we report the generation and the characterization of an *ezh2*-deficient zebrafish line. In contrast to its essential role in mouse early development, loss of *ezh2* function does not affect zebrafish gastrulation. *Ezh2* zebrafish mutants present a normal body plan but die at around 12 dpf with defects in the intestine wall, due to enhanced cell death. Thus, *ezh2*-deficient zebrafish can initiate differentiation toward the different developmental lineages but fail to maintain the intestinal homeostasis. Expression studies revealed that *ezh2* mRNAs are maternally deposited. Then, *ezh2* is ubiquitously expressed in the anterior part of the embryos at 24 hpf, but its expression becomes restricted to specific regions at later developmental stages. Pharmacological inhibition of Ezh2 showed that maternal Ezh2 products contribute to early development but are dispensable to body plan formation. In addition, *ezh2*-deficient mutants fail to properly regenerate their spinal cord after caudal fin transection suggesting that Ezh2 and H3K27me3 methylation might also be involved in the process of regeneration in zebrafish.

1. Introduction

Polycomb-group (PcG) proteins are epigenetic repressors of transcriptional programs involved in the maintenance of cellular identity during development and differentiation [1–3]. PcG proteins assemble into at least two well-characterized and biochemically distinct chromatin-modifying protein complexes that are termed Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). In the canonical pathway, PRC2 composed of the core proteins EZH2, EED and SUZ12 together with RBAP46/48 and other accessory subunits, initiates gene silencing by catalyzing di- and trimethylation of lysine 27 of histone H3 (H3K27me2/3) [4–8]. PRC1 is then recruited to the target regions by binding to H3K27me3 marks through the CBX component of PRC1 [9], and subsequently catalyzes the monoubiquitinylation of H2AK119 (H2AK119ub1) to maintain gene silencing [10–12].

Within the PCR2, EZH2 is a SET domain-containing protein harboring the histone methyltranferase activity, whereas EED and SUZ12 are involved in PRC2 stability and are necessary for EZH2 catalytic activity [13–15]. Genome-wide studies in mouse and human embryonic stem cells revealed that PRC2 and H3K27me3 marks are deposited at the promoters of numerous genes involved in cell differentiation, lineage specification and development, leading to the idea that EZH2 may be involved in the maintenance of the pluripotency of stem cells by keeping developmental genes repressed [9,16–22]. Differentiation would then be associated with a relocation of PRC2, in turn responsible for the repression of the stem cell genes and enabling activation of gene expression programs specific to given developmental lineages. Indeed, the PRC2 components are essential for early mouse development and knock-out mutants for *Ezh2*, *Eed* and *Suz12* initiate but fail to complete gastrulation and die at around embryonic days 7 to 9 [14,23,24]. This death correlates with the alteration of lineage-specifying gene expression, a decrease in cell proliferation and an increase

Abbreviations: bp, base pair; dpf, days post fertilization; dpa, days post amputation; ezh2, enhancer of zeste homolog 2 (polycomb repressive complex 2 subunit); hpf, hours post fertilization; gcga, glucagon a; ins, insulin; NMD, nonsense-mediated decay; nt, nucleotide; phox2bb, paired-like homeobox 2bb; PRC, Polycomb Repressive Complex; rnf2, ring finger protein 2; TALEN, transcription activator-like effector nuclease; Try, trypsin

* Corresponding author at: Cell Plasticity & Cancer, Inserm U908/University of Lille, Bâtiment SN3, Cité Scientifique, F-59655 Villeneuve d'Ascq, France.
E-mail address: pierre-olivier.angrand@univ-lille1.fr (P.-O. Angrand).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbagrm.2017.08.011
Received 3 May 2017; Received in revised form 16 August 2017; Accepted 27 August 2017
Available online 05 September 2017
1874-9399/

of apoptosis [14].

In human, recent cancer genome sequencing and expression studies reported that several genes encoding PRC2 subunits are mutated or have their expression altered in different cancer types [25,26]. Furthermore, mutations in EZH2 were shown to cause the Weaver syndrome (OMIM:277590), a syndrome characterized by skeletal overgrowth, tall stature, a dysmorphic craniofacial appearance and variable intellectual disability [27–30].

The zebrafish model provides a unique tool to investigate gene function during development . The zebrafish (*Danio rerio*) is a widely used vertebrate model for studying development and morphogenesis. Owing to external fertilization and optical transparency of the embryos, early development of zebrafish can be easily monitored. Furthermore, the recent emergence of powerful genome-editing technologies, such as the Transcription Activator-Like Effector Nuclease (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated System (CRISPR/Cas9) applied to zebrafish allows rapid gene function studies in this organism [31–36]. Using the TALEN technology, we generated here a heterozygous zebrafish line harboring an *ezh2* loss-of-function allele to investigate its role in development. We show that in contrast to what is observed in mice, *ezh2* zygotic expression is not required for gastrulation and tissue specification in zebrafish. However, homozygous mutants die at about 12 dpf with defects in the intestine wall, suggesting that zygotic *ezh2* expression is necessary for the maintenance of the integrity of the intestine at later developmental time points. Furthermore, we show that the ability of spinal cord regeneration at the caudal fin is also affected in homozygous mutant larvae.

2. Materials and methods

2.1. Zebrafish maintenance, embryo preparation and treatment

Zebrafish (TU strain) were maintained at 27.5 °C in a 14/10 h light/dark cycle. The evening before spawning, males and females were separated into individual tanks. Spontaneous spawning occurred when the light turned on and embryos or larvae were collected and staged according to Kimmel et al. [37]. The feeding of zebrafish larvae starts at 6 dpf for all experiments. The chorions were removed from embryos by the action of 1% pronase (sigma) for 1 min. Zebrafish embryos or larvae were fixed overnight in 4% paraformaldehyde in PBS (phosphate-buffered saline, Invitrogen), dehydrated gradually to 100% methanol and kept at − 20 °C.

For caudal fin amputation, 6-month-old adult zebrafish were anesthetized with MS-222 (tricaine, ethyl 3-aminobenzoate methanesulphonate, 250 mg/L; Sigma-Aldrich) and approximately two-thirds of the fin was cut with a blade. After amputation, fish were placed in the aquarium at 27.5 °C for fin regeneration. The blastemal starts to form at approximately 24 h post amputation (hpa) and the amputated fins have been fully restored at around 20 days post amputation (dpa). Larval caudal fin transections were performed at 3 dpf within the pigment gap distal to the circulating blood after anesthesia, as described by Wilkinson et al. [38].

To inhibit Ezh2 activity, dechorionated embryos were exposed to 1 μM GSK126 (A11757, Adooq Bioscience) dissolved in DMSO or to an equivalent concentration of DMSO (0.01%, control) at the 1–2 cell stage, at 3 hpf or after larval caudal fin amputation.

2.2. Animal ethics statement

The zebrafish experiments described in this study were conducted according to the French and European Union guidelines for the handling of laboratory animals (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). The experimental procedures carried out on zebrafish were reviewed and approved by the local Ethics Committee of the Animal Care Facility of the University of Lille. At the end of the experiment, fish older than 8 dpf were humanely euthanized by immersion in an overdose of tricaine methane sulfonate (MS-222, 300 mg/L) for at least 10 min, whereas younger fish were immobilized by submersion in ice water (5 parts ice/1 part water, 0–4 °C) for at least 1 h to ensure death by hypoxia.

2.3. TALEN design and assembly. The ezh2.

TALEN target site was selected using the online TAL Effector-Nucleotide Targeter tool (https://tale-nt.cac.cornell.edu/; [39]) in exon 2 with the following parameters: (i) spacer length of 14–17 bp, (ii) repeat array length of 16–18 bp, (iii) each binding site was anchored by a preceding T base in position “0” as has been shown to be optimal for naturally occurring TAL proteins [40,41], (iv) presence of a restriction site (DdeI) within the spacer sequence for screening and genotyping purposes.

Ezh2-specific TALEN constructs were engineered using the TALEN Golden Gate assembly system described by Cermak et al., [42]. The TALEN expression backbones, pCS2TAL3DD and pCS2TAL3RR [31], and the plasmids providing repeat variable diresidues (RVD) [42] for Golden Gate Cloning were obtained from Addgene.

2.4. mRNA injection into zebrafish embryos

Capped mRNAs were synthetized using the SP6 mMESSAGE mMACHINE kit (Ambion) from linearized plasmid templates. mRNAs (50–100 pg) were injected into 1-cell zebrafish embryos using a FemtoJet microinjector (Eppendorf).

2.5. Genotype analyses

Three-days-old embryos or pieces of caudal fin were incubated in 20 μL PCR extraction buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.2% Triton X-100, 100 μg/mL proteinase K) and placed at 50 °C for 4 h prior proteinase K inactivation at 95 °C for 5 min. Genotype analysis was performed by PCR on 2.5 μL of samples using the primer set TAL_ezh2_5′_S21Ac (GGTATGGTTGTTGCAGTTCACAGAC) and TAL_ezh2_3′_S21Ac (AACACCAAACTCTACACAAGCAGCA) followed by PCR product digestion with the DdeI restriction enzyme. Sequence determination (GATC-biotech, Germany) was performed after cloning of the PCR products into pCR-XL-TOPO (Invitrogen) according to the manufacturer's instructions

To achieve genotyping on paraformaldehyde-fixed embryos and larvae, DNA was extracted using sodium hydroxide and Tris [43]. Briefly, single embryos were placed into microcentrifuge tubes containing 20 μL 50 mM NaOH and heated 20 min at 95 °C. The tubes were then cooled to 4 °C and 2 μL of 1 M Tris-HCl, pH 7.4 was added to neutralize the basic solution. Genotype analysis was performed on 2.5 μL of samples by PCR-DdeI digestion, as described above.

2.6. Alcian blue staining

Alcian blue staining was performed as previously described [44]. Zebrafish larvae were fixed 2 h at room temperature in 4% paraformaldehyde and dehydrated 10 min in 50% ethanol. Cartilages were stained in 0.02% Alcian blue (Sigma Aldrich), 60 mM $MgCl_2$, 70% ethanol, overnight at room temperature. Pigments were bleached by a 2-hour incubation in water containing 1% KOH, 3% H_2O_2. Larvae were then digested with 0.05% trypsin (Sigma Aldrich) until tissue disappeared (around 4 h) before storage in 70% glycerol. After imaging with a Leica MZ125 stereomicroscope equipped with a Leica DFC295 digital camera, DNA was extracted using sodium hydroxide and Tris for genotyping purposes.