Chromatin remodeling factor CECR2 forms tissue-specific complexes with CCAR2 and LUZP1

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Chromatin remodeling factor CECR2 forms tissue-specific complexes with CCAR2 and
LUZP1

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Abstract

Chromatin remodeling complexes alter chromatin structure to control access to DNA and therefore control cellular processes such as transcription, DNA replication, and DNA repair. CECR2 is a chromatin remodeling factor that plays an important role in neural tube closure and reproduction. Loss-of-function mutations in *Cecr2* result primarily in the perinatal lethal neural tube defect exencephaly, with non-penetrant mice that survive to adulthood exhibiting subfertility. CECR2 forms a complex with ISWI proteins SMARCA5 and/or SMARCA1, but further information on the structure and function of the complex is not known. We therefore have identified candidate components of the CECR2-containing remodeling factor (CERF) complex in embryonic stem (ES) cells through mass spectroscopy. Both SMARCA5 and SMARCA1 were confirmed to be present in CERF complexes in ES cells and testis. However, novel proteins CCAR2 and LUZP1 are CERF components in ES cells but not testis. This tissue specificity in mice suggests these complexes may also have functional differences. Furthermore, LUZP1, loss of which is also associated with exencephaly, appears to play a role in stabilizing the CERF complex in ES cells.

Keywords: CECR2, LUZP1, CCAR2, Chromatin remodeling factor, Neural tube defects

1 Introduction

2
3 The compact structure of chromatin makes genomic DNA inaccessible for DNA-binding
4 factors involved in many important cellular processes such as transcription, DNA replication,
5 DNA repair, homologous recombination and chromatin assembly (Hargreaves and Crabtree,
6 2011). All eukaryotic cells use large multiprotein complexes to control these crucial
7 biological processes (Piatti et al., 2011). ATP-dependent chromatin remodeling complexes
8 function through energy produced from ATP hydrolysis to alter histone-DNA interactions
9 within the nucleosomes, making protein-binding sites accessible or inaccessible to DNA-
10 binding proteins (Varga-Weisz, 2010, p.). In addition to an ATPase, these complexes can
11 contain multiple non-catalytic components that influence complex remodeling functions and
12 are required for targeting them to specific genomic loci (Bartholomew, 2014).

13 The mammalian ISWI (imitation switch) family of ATP-dependent chromatin
14 remodeling complexes contains either SMARCA1 (SNF2L) or SMARCA5 (SNF2H) as their
15 ATPase catalytic components (Oppikofer et al., 2017). An example is the CECR2-containing
16 remodeling factor (CERF) complex. Reduction or loss of the defining component, CECR2, in
17 mice results in the lethal neural tube defect exencephaly (Leduc et al., 2017). Homozygous
18 *Cecr2* mutant mice that are non-penetrant for exencephaly survive into adulthood but are
19 subfertile, producing significantly smaller litters than wild-type mice (Norton et al., 2020;
20 Thompson et al., 2012). Thus, CERF functions in both neurulation and reproduction.

21 While we previously showed interaction between CECR2 and SMARCA5 in mouse
22 ES cells and adult mouse testis (Thompson et al., 2012), the additional members of the CERF
23 complex are unknown. Using mass spectrometry, immunoprecipitation, and
24 immunofluorescence we identified additional complex components. We show that both
25 CCAR2 and LUZP1 are novel members of the CERF complex, but in a tissue-specific
26 manner, being present in ES cells but not testis. *Luzp1* is known to be involved in neurulation

and its loss in mice results in exencephaly (Hsu et al., 2008). Here we show evidence that in ES cells, LUZP1 acts to stabilize the CERF complex.

2. Materials and methods

Cell culture, mouse tissues.

TT2 (Yagi et al., 1993), E14 and Luzp-KO/lacZ-KI (Lee et al., 2001) mouse ES cells were cultured as previously described (Thompson et al., 2012). Derivation and culture of primary neural stem cells (neurospheres) is described in Elliott et al (2020). BALB/cCrAlt mice for testis tissue were reared and genotyped as previously described (Thompson et al., 2012). The *Cecr2* mutant mice used as a control for immunofluorescence were compound heterozygotes for a hypomorphic genetrap mutation (*Cecr2^{Gt(pGT1)1Hemc}*, here referred to as *Cecr2^{GT}*) (Banting et al., 2005) and a presumptive null deletion mutation (*Cecr2^{tm.1.1Hemc}*, here referred to as *Cecr2^{Del}*) (Fairbridge et al., 2010). Approval for all experiments using mice was obtained from the Animal Care and Use Committee of the University of Alberta (AUP 00000094) and conformed to guidelines of the Canadian Council on Animal Care.

Western blotting

ES cells and wild-type adult mouse testes were homogenized in lysis buffer (150 mM sodium chloride, 1.0% IGEPAL CA-630, 10% Glycerol, 2 mM EDTA and 20 mM Tris pH 8.0) supplemented with protease inhibitors. The mixture was rocked for 30 min at 4°C and the protein extract was cleared by centrifugation. Protein quantification and Western blotting was performed as previously described (Thompson et al., 2012). Primary antibodies were rabbit polyclonal anti-CECR2 (1:50,000)(Norton et al., 2020), rabbit polyclonal anti-SMARCA5 (1:2000, Abcam), rabbit polyclonal anti-LUZP1 (1:5000; Protein tech), rabbit

polyclonal anti-CCAR2 (1:1000; Protein tech), sheep polyclonal anti-SMARCA1 (1:1000; provided by Dr. David Picketts). Secondary antibodies were goat anti-rabbit IgG-HRP (Bio-Rad) and rabbit anti-sheep IgG-HRP (Santa Cruz).

Immunoprecipitation (IP)

Approximately 1 µg of antibody was rocked with 10 µl of Dynabeads® Protein A (Life Technologies) in 200 µl of PBS containing 0.02% Tween-20 at room temperature for each IP reaction. Approximately 500-1000 µg of whole cell lysate was diluted with IP dilution buffer (50 mM Tris pH 8.0, 1% IGEPAL® CA-630) to a final concentration of ~150 mM NaCl and added to the antibody-beads mixture and incubated overnight at 4°C. Following the incubation, beads were washed with lysis buffer containing 0.1% Tween-20. To elute IP'ed proteins, beads were then heated with 10 µl of 4x NuPAGE™ loading buffer (Life Technologies) and 10 µl of 1 M β-mercaptoethanol at 95-100°C for 5 minutes. The supernatant containing the eluted proteins was separated from the beads and analyzed by Western blot.

Mass spectrometry

Four biological replicate experiments from independent cell cultures were used. Immunoprecipitated protein samples were subjected to SDS-PAGE and run approximately 10 mm into a 4-20% gel (Bio-Rad). After staining with Coomassie Blue, the gel piece containing all the proteins was cut into bands with a width of ~5 mm. In one of the replicates only bands between ~130 kDa and ~200 kDa were excised from the gel. To prepare samples for mass spectrometry, the In-Gel Tryptic Digestion Kit (Thermo Scientific) was used. Nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to an LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific) was used to resolve and ionize the tryptic peptides.

Analysis of the mass spectrometric data was performed using Proteome Discoverer 1.4 (Thermo Scientific). The generated result was searched against Uniprot (uniprot.org) Mus database using SEQUEST (Thermo Scientific) with the parameters including a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. All searches set the static modification as Carbamidomethyl cysteine and dynamic modifications as oxidized methionine and deamidated glutamine and asparagine as dynamic modifications. Gene ontology analysis was done using PANTHER database (<http://www.pantherdb.org/>).

Gel filtration

Gel filtration was carried out as previously described (Thompson et al., 2012) with a few modifications. A Sephacryl S400 HR column was equilibrated in 150 mM sodium chloride, 1.0% IGEPAL CA-630, 20 mM Tris pH 8.0 and 2 mM EDTA. Approximately 1 mg of whole cell lysate was loaded onto the column and 0.5 ml fractions were collected at a flow rate of 0.5 ml/min. The peak CECR2-containing fractions were precipitated and pooled for use in IP as described above.

Immunofluorescence staining

Testes from adult male *Cecr2*^{+/+} and *Cecr2*^{GT/Del} mice were fixed in 4% formaldehyde in PBS for up to 48 hrs at room temperature (RT), then embedded in paraffin and sectioned. Slides boiled for 10 minutes in Antigen Retrieval Buffer (10 mM Tr Base, 1 mM EDTA, 0.05% Tween 20) four times. Sections were blocked in 10% normal goat serum (Sigma) and 0.6% Triton-X (Sigma) diluted in PBS for 1 hour at RT, then diluted primary antibody overnight at 4°C in a humidifying chamber (Antibody Dilution Buffer: 0.1% bovine serum albumin, 0.3% Triton-X, diluted in PBS). Primary antibody concentrations used were:

purified rabbit polyclonal anti-CECR2 (1:10 000), and rabbit polyclonal anti-CCAR2 (Protein tech #22638-1-AP) (1:1000).

Sections were then washed in PBS and incubated in Alexa Fluor® 488 AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) reconstituted in water at a 1X concentration for 2 hours at RT in the dark. Sections were washed in PBS and blocked in 20% normal goat serum and 0.6% Triton-X diluted in PBS for 1 hour followed by incubation in the second diluted primary antibody in a humidifying chamber for 2 hours. Sections were washed three times in PBS for 5 minutes each, and then incubated in diluted secondary antibody (Goat anti-Rabbit IgG (H+L) Secondary Antibody-Alexa Fluor 555) at 1:200 in a humidifying chamber for 2 hours. Sections were stained with 0.1% DAPI in PBS for 5 minutes and washed again in PBS three times for 5 minutes each. Coverslips were mounted onto slides using Fluoromount G (SouthernBiotech) and sealed before being imaged with the Nikon Eclipse 80i confocal microscope (CVI Melles Griot Ion Laser, NIS-Elements v4.0 software).

3. Results

Identifying additional CECR2 interacting proteins using mass spectroscopy

ISWI complexes often have many interacting protein components. For instance, CHRAC and NuRD/cohesin have 4 and 10+ components, respectively (Dirscherl and Krebs, 2004). To investigate the composition of CERF complexes in ES cells, an IP approach from biological replicates of cell lysates was used, followed by LC-MS/MS mass spectrometry (Table S1-4). To eliminate non-specific binding proteins, normal rabbit IgG was used in parallel IP reactions and proteins found on both IPs were eliminated. Two criteria were considered to determine a list of novel candidate CECR2-interacting proteins for further study: (1) identification of the protein in more than one mass spectrometry experiment and (2) the protein having a biological function related to CECR2, such as processing chromatin

binding, having DNA binding properties or involvement in neural tube defects. In mice the neural tube begins to close at ~E8.5. Lack of closure in the cranial region results in exencephaly (anencephaly in humans) and failure of the brain to form properly (Copp et al., 2003). Over 300 genes are known to be involved in neural tube closure, including chromatin remodellers (Wilde et al., 2014). A visualization of the protein classes, biological processes and molecular function of 141 candidate CECR2-interacting proteins are shown in Fig. S1. The top 10 candidate proteins are given in Table 1. Both SMARCA1 and SMARCA5 were present as expected (Banting et al., 2005; Oppikofer et al., 2017; Thompson et al., 2012). Since we previously showed that *Cecr2* has a role in male reproduction (Thompson et al., 2012), we also investigated *Cecr2* interactions in the testis. Using IP, we confirmed the interaction of native CECR2 with the two SMARCA proteins from whole cell lysates of both ES cells and adult testes (Fig. 1a-d). We then focused on novel proteins CCAR2 and LUZP1.

CCAR2 interacts with CECR2 in ES cells but not testis

Cell Cycle and Apoptosis Regulator 2 (CCAR2), formerly known as Deleted in Breast Cancer 1 (DBC1) and DBIRD Complex Subunit KIAA1967, participates in a wide range of functions (Magni et al., 2018) which could be associated with chromatin remodeling. Reciprocal IP assays confirmed the interaction between CECR2 and CCAR2 in ES cells (Fig. 2a). Unexpectedly in testis, reciprocal IP assays indicated a lack of interaction (Fig. 2b). We therefore performed immunofluorescence on ES cells and testis sections to see if the two proteins colocalized (Fig. 2c-f and 2g-j). The results showed considerable colocalization of CECR2 and CCAR2 in the nuclei of ES cells, which is consistent with the IP results (Fig 2c-f). The results of immunofluorescence on testis sections showed that CECR2 localized to spermatogonial cells in the seminiferous tubule periphery (Fig. 2h) while CCAR2 localized to other cells in the periphery and more differentiated cells closer to the lumen, (Fig. 2i).

CCAR2-positive cells showed no overlap with CECR2-positive cells (Fig. 2j). This lack of colocalization supports a lack of interaction between CECR2 and CCAR2 in the testis as seen with the IP results.

LUZP1 interacts with CECR2 in ES cells but not testis

The *Luzp1* gene is involved in neural tube closure and similar to *Cecr2* produces an exencephaly phenotype when mutated (Hsu et al., 2008), making LUZP1 a strong candidate for being a CERF complex member. Reciprocal IP assays using whole cell lysates confirmed the interaction of LUZP1 and CECR2 in ES cells, (Fig. 3a) but similar to CCAR2, not in testes (Fig. 3b). Reciprocal co-IPs also showed an interaction between SMARCA5 and LUZP1 in ES cells, as would be expected for CERF, but could also represent other ISWI complexes containing SMARCA5 (Fig. 3c). However, IP from LUZP1-deficient ES cells showed that CECR2 and SMARCA5 interact with each other in the absence of LUZP1 and therefore the presence of LUZP1 is not necessary for the SMARCA5-CECR2 interaction (Fig. 3d). Since loss of LUZP1 results in exencephaly (Hsu et al., 2008), we tested and found an interaction between LUZP1 and CECR2 in neurospheres (Fig. 3e), which could suggest these two proteins work together during neurulation.

To further investigate the presence of LUZP1 in the CERF complex in ES cells, gel filtration was performed. CECR2 eluted mostly in 3-5 high molecular weight fractions, suggesting a complex of approximately 1.6- 2 MDa (Fig. 3f,h). Co-IPs of the largest two fractions containing CECR2 were shown to also contain SMARCA5 and LUZP1(Fig. 3g and i respectively), supporting the presence of LUZP1 in the 2 MDa CERF complexes in ES cells.

LUZP1 acts as a bridging protein in the CERF complex

Krebs et al (2010) showed that LUZP1 is part of the molecular bridge connecting complexes ATAC and MED in mouse ES cells to create the meta-coactivator (MECO) complex, and that lack of LUZP1 resulted in partial disassembly of ~ 50% of MECO complexes (Krebs et al., 2010). To assess whether LUZP1 could be playing a similar role in the CERF complex in ES cells, whole cell lysates prepared from wild-type and *Luzp1* mutant ES cells (E14 and *Luzp*-KO/*lacZ*-KI respectively) were fractionated using a gel-filtration column. Western blot analysis of the eluted fractions showed the presence of the ~1.6-2 MDa CERF complex (fractions 26-32) in wild-type ES cells as expected (Fig. 3j). However, in the *Luzp*-KO/*lacZ*-KI ES cells there was a dramatic decrease in the larger-sized complexes and an increase in smaller-sized complexes (Fig. 3k). Although there is still a CERF complex detected at ~1.6-2 MDa, most of the CECR2 protein is eluted in fractions 35-43, corresponding to sizes of ~400 kDa-1.4 MDa. Elution of CECR2 in a broad range of mobilities in the absence of LUZP1 suggested that LUZP1 has a role in stabilizing CERF complexes. CERF is not likely to be complexed with either the ATAC or MED complexes, since none of the components of either complex were seen in any of the CERF complex mass spectrometry experiments.

Discussion

Mammalian ISWI complexes, including CERF, are multi-protein complexes containing SMARCA1 or SMARCA5 as the catalytic interacting protein (Erdel and Rippe, 2011). Our previous analysis indicated that a CECR2^{GT}/*LacZ* fusion protein is part of a ~300-400 kDa CERF complex with SMARCA5 in mouse ES cells (Thompson et al., 2012). However, because the fusion protein contains only 20% of the CECR2 protein and the experiments were done at a high salt concentration (420 mM), these factors could affect the ability of the fusion protein to form normal CERF complexes with other proteins. Here we

1 show that native CECR2 complexes form with both SMARCA1 and SMARCA5 in ES cells
2 and adult testis tissue, and that the ES complex at physiological salt concentration is ~1.6-2
3 MDa, suggesting the presence of additional interacting proteins.

4 Mass spectroscopy analysis of CECR2 showed a number of possible candidate CERF
5 complex proteins, of which we studied two, both of which show tissue specificity. CCAR2 is
6 a component of numerous complexes with different functions, including regulation of
7 transcription, apoptosis, and double-stranded DNA break repair (Magni et al., 2018). Our IP
8 results showed that CCAR2 interacts with CECR2 in ES cells but not in testis. IF showed that
9 although CCAR2 is present in the testis, there was no detectable colocalization with CECR2,
10 supporting that the two proteins do not interact. LUZP1 is a member of the MECO complex
11 (Krebs et al., 2010) and has recently also been found to function as an actin crosslinking
12 protein (Wang and Nakamura, 2019) and a cilia regulator (Bozal-Basterra et al., 2020;
13 Gonçalves et al., 2020). We found LUZP1 in CERF complexes in mouse ES cells and
14 neurospheres, but not in testis. The presence of LUZP1 in neurosphere CERF complexes is
15 particularly interesting, since the loss of *Cecr2* or *Luzp1* in mice results in exencephaly (Hsu
16 et al., 2008). It would be interesting to look at other tissues that express CECR2 to see if
17 patterns arise with respect to different CERF compositions.

18 LUZP1 is part of the molecular bridge that connects the ATAC complex to the MED
19 complex forming the MECO complex in mouse ES cells (Krebs et al., 2010). The lack of
20 LUZP1 leads to partial dissociation of the MECO complex (Krebs et al., 2010). Similarly, the
21 lack of LUZP1 leads to partial dissociation of the CERF complex in ES cells, resulting in two
22 broad peaks in the gel filtration profile of CECR2. This suggests that the presence of LUZP1
23 contributes to the stability of CECR2 complexes in ES cells. Similar to MECO, other proteins
24 may also be involved in stabilization since a significant amount of the ~2 MDa complex
25 remained. None of the known components of the ATAC or MED complexes were detected in

the mass spectrometry analyses, suggesting that LUZP1 is not mediating the interaction of one of these complexes with CERF. Further investigation is needed to elucidate the intricacies of the CERF complex compositions in different tissues. This tissue-specific diversity in the CERF complex is suggestive of tissue-specific functions.

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Competing interests statement

The authors declare there are no competing interests.

Contributors' statement

F.N. - Investigation, writing-original draft

A.T. - Investigation, writing-review & editing

K.R.L. - Investigation, writing- review & editing

H.M. - Supervision, writing & editing

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Table 1. List of the top 10 candidate CECR2 interacting proteins in ES cells. Proteins were identified using LC-MS/MS on complexes IP'ed with the anti-CECR2 antibody. Three independent biological replicates that included the whole gel were performed separately and combined. Protein order in the list is based on decreasing average score. Bolded proteins were further examined.

TOP2A	99	15
SMARCA1	89	9
SMARCA5	88	10
CCAR2	60	9
CECR2	43	10
LUZP1	28	7
THRAP3	17	4
TMOD3	15	4
MATR3	13	3
RUVBL1	7	3
DNTTIP2	7	3

*Also known as DBIRD complex subunit KIAA1967 homolog

Figure legends

Fig. 1. CECR2 forms the CERF complex with SMARCA 1 and SMARCA5 in mice. Co-IP was performed to support the mass spectroscopy data. As expected, SMARCA1 and SMARCA5 showed interaction with CECR2. CECR2 and SMARCA1 interacted in whole cell lysates from ES cells (a) as well as testis tissue (b) as shown by Western blot analyses following IP with anti-CECR2 antibody or rabbit IgG (CTRL). The SMARCA1 antibody did not allow a reciprocal co-IP to be done. CECR2 and SMARCA5 also interacted in ES cells (c) and adult testis (d). In all experiments the input represents 5-10% of the extract used in the IP reaction. Approximately 20-25% of each IP reaction was used in each Western blot analysis. The molecular weight standards are shown as kDa on the left side of the figure.

Fig. 2. The CERF complex contains novel protein CCAR2 in ES cells but not in testis tissue.

Reciprocal Co-IP experiments showed an interaction between CECR2 and CCAR2 in ES cells (a) but not in testes (b). Immunofluorescence staining on ES cells showed that CECR2 and CCAR2 colocalize (c-f). The overlapping CECR2 and CCAR2 staining is seen as white in the CCAR2/CECR2 merge image (f). Supporting the lack of interaction in testis, immunofluorescence staining on testis paraffin sections showed that CECR2 and CCAR2 did not colocalize (g-j). CECR2 was localized to a subset of spermatogonia (h, arrowheads), while CCAR2 was localized to different peripheral cells and cells closer to the lumen. DAPI staining shows the nuclei. IP parameters are as described in Figure 1.

Figure 3. The CERF complex contains novel protein LUZP1 in neurospheres and ES cells, where LUZP1 has a role in the stability of CERF complex.

Reciprocal IP analysis of whole cell lysates showed an interaction between CECR2 and LUZP1 in ES cells (E14 cell line) (a). CECR2 did not interact with LUZP1 in adult testis (b). LUZP1 and SMARCA5 also reciprocally co-IP'ed in ES cells (c). Western blot analysis of LUZP1 in both wild type ES cells (E14, LUZP1+/+) and homozygous Luzp-KO/lacZ-KI ES (LUZP1-/-) cells shows the mutant line had no detectable LUZP present (d, left panel). CECR2 interacted with SMARCA5 in both LUZP1+/+ wild type ES cells and Luzp-KO/lacZ-KI ES (LUZP1-/-) mutant cells in the absence of LUZP1, showing that their interaction is not LUZP1 dependent (d). LUZP1 and CECR2 were also shown to co-IP in neurospheres (e). Colocalization analysis using the CECR2 and LUZP1 antibodies by

1 immunofluorescence was not technically possible. Elution profiles of CERF complexes were
2 produced through gel filtration. The complexes in ES cells ranged from ~1.6 to 2 MDa
3 (f,h,and j), with a peak at ~ 2 MDa in Fraction 24 of panel f. Both SMARCA5 (g) and
4 LUZP1 (h,i) co-IP'ed with CECR2 from gel filtration fractions corresponding to the peak of
5 CECR2 elution. Gel filtration elution profile of the CERF complex of wild type (j), with the
6 peak fractions of 26-32 corresponding to molecular weight of 1.6-2 MDa, and homozygous
7 Luzp1 mutant (k) ES cell lines with the peak fractions of 35-43 corresponding to molecular
8 weight of 400 KDa-1.4 MDa showed that the size of much of the CECR2 complex became
9 smaller in the absence of LUZP1. IP parameters are as described in Figure 1. INPUT in gel
10 filtrations represents ~1% of the extract loaded on the column. V0 is the void volume of the
11 column. Tubulin was added as a loading control.

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Fig. 1

Fig. 3