FET family fusion oncoproteins target the SWI/SNF chromatin remodeling complex

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Abstract

Members of the human FET family of RNA-binding proteins, comprising FUS, EWSR1, and TAF15, are ubiquitously expressed and engage at several levels of gene regulation. Many sarcomas and leukemias are characterized by the expression of fusion oncogenes with FET genes as 5’ partners and alternative transcription factor-coding genes as 3’ partners. Here, we report that the N terminus of normal FET proteins and their oncogenic fusion counterparts interact with the SWI/SNF chromatin remodeling complex. In contrast to normal FET proteins, increased fractions of FET oncoproteins bind SWI/SNF, indicating a deregulated and enhanced interaction in cancer. Forced expression of FET oncogenes caused changes of global H3K27 trimethylation levels, accompanied by altered gene expression patterns suggesting a shift in the antagonistic balance between SWI/SNF and repressive polycomb group complexes. Thus, deregulation of SWI/SNF activity could provide a unifying pathogenic mechanism for the large group of tumors caused by FET fusion oncogenes. These results may help to develop common strategies for therapy.

Keywords EWSR1-FLI1; FET proteins; FUS-DDIT3; fusion oncogenes; SWI/SNF chromatin remodeling complex

Subject Categories Cancer; Chromatin, Epigenetics, Genomics & Functional Genomics; Transcription

DOI 10.15252/embr.201845766 | Received 14 January 2018 | Revised 6 March 2019 | Accepted 11 March 2019 | Published online 8 April 2019
EMBO Reports (2019) 20: e45766

Introduction

The FET family genes FUS, EWSR1, and TAF15 (also known as TLS, EWS, and TAF2N, respectively) encode RNA-binding proteins (Fig 1A) that are proposed to link transcription with the subsequent steps of RNA splicing, processing and transport [1–5], localized translation [6], and micro-RNA processing [7]. Fusion oncogenes with FET genes as 5’ partners and alternative transcription factor-coding genes as 3’ partners (Fig 1B) are pathognomonic of many types of sarcoma and leukemia [8,9]. FET-oncogene-caused tumors contain few other mutations, indicating that FET fusion oncogenes impact on crucial mechanisms in tumor development [9–15].

FET fusion oncogenes invariably contain the N-terminal domains (NTDs) of the FET partners juxtaposed to DNA-binding parts of the transcription factor partners (Fig 1A and B). They are reported to act as aberrant transcription factors with the NTDs as strong trans-activator domains [16–25]. Forced expression or silencing of FET oncogenes affects tumor morphology and regulation of large numbers of genes, and changes the epigenetic landscape [24–27].

The three normal FET proteins contain central RNA recognition motifs (RRM) flanked by RGG repeat regions and potential single-stranded DNA- or RNA-binding zinc finger domains (Fig 1A) [28–30]. The NTDs largely consist of structurally disordered, prion-like degenerated SYGQ-rich repeats, and their compositions suggest functions in protein–protein interactions [31]. Their similarities are further underscored by the fact that they functionally replace each other as N-terminal partners in some FET fusion oncoproteins and tumor entities [28,32]. Given these observations, we hypothesized that the three FET-NTDs could act by binding the same key interaction partner. However, even with several FET-binding proteins identified, no such interaction partners have been reported and the role of the NTDs remains enigmatic. The aim of this study was to identify major interaction partners shared by the three FET-NTDs that might give clues to a common pathogenetic mechanism.

Results and Discussion

The FET-NTDs mediate binding of both normal and oncogenic FET proteins to SWI/SNF

We used bacterially expressed GST-tagged recombinant constructs in pulldown experiments with cell extracts for an unbiased analysis
of enrichment of FET-NTD-binding proteins (Fig 1A). SDS-PAGE analysis and protein staining revealed several high-molecular-weight proteins captured by all three FET-NTDs (Fig 1C). Mass spectrometry analysis (MS) of excised gel bands identified peptides from core components of the SWI/SNF chromatin remodeling complex: ARID1A (BAF250A), BRG1 (SMARCA4), BAF170, and others.
Figure 1. Identification of proteins bound to recombinant FET-NTD baits.

A The three normal RNA-binding FET proteins consist of N-terminal repetitive and structurally disordered domains (NTD: N-terminal domain), central RNA-binding domains (RRM: RNA recognition motifs), zinc finger domains (ZF), and degenerated repeat regions (RGG: RGG repeat regions). The GST-tagged FET-NTD baits shown were used for pulldown experiments and represent the shortest parts commonly present in the FET fusion oncoproteins. The FET-binding motif is a conserved sequence required for complex formation between the three FET proteins. Amino acid numbers are indicated.

B Schematic illustration of a representative FET fusion protein (FUS-DDIT3 type II) and its parental proteins. DBD: DNA-binding domain, LZ: leucine zipper domain. Type I and type II show locations of the two most common MLS fusion breakpoints in FUS.

C Coomassie staining of SDS–PAGE-separated pulldown samples with/without cell extracts (C.E) using GST-tagged FET-NTDs as baits. Sepharose with bound GST was included as control. Background from recombinant protein baits (shown by white arrowheads) as well as smaller partial recombinant products is visualized in the left panel (C.E −). Several high-molecular-weight proteins are retained by the FET-NTDs (C.E +). Black arrowheads indicate protein bands and gel parts analyzed by mass spectrometry (see also Table EV1).

D Immunoblot analysis (IB) of pulldown samples with FET-NTD baits with/without cell extracts (C.E). Antibodies against ARID1A, BRG1, and BAF60A were used for detection of SWI/SNF components. Input samples diluted 1:5 were included as a control.

E In situ proximity ligation assays (PLA) using antibodies against BRG1, ARID1A, DDIT3, and C-terminal parts of normal FUS show protein complexes containing FUS/BRG1, FUS/ARID1A, FUS-DDIT3/BRG1, and FUS-DDIT3/ARID1A as red fluorescent spots in nuclei of MLS cell lines 2645-94 and 402-91. C-terminal parts of FUS are not present in the FUS-DDIT3 fusion protein, and normal DDIT3 is not expressed in these cell lines. Merged images also include DAPI nuclear counterstain in blue. Combinations of primary antibodies are used to detect interactions (left panel). In control experiments (right panel), one primary antibody is omitted to evaluate the background fluorescent signals. Scale bars = 10 μm.

Source data are available online for this figure.
BRG1-precipitated complexes indicating a dysregulated interaction with SWI/SNF. Furthermore, gentle formic acid elution released substantial amounts of EWSR1 whereas elution with the strong LDS detergent was needed to release the FUS-DDIT3 fusion protein from anti-BRG1-captured complexes (Fig EV1A and B). Taken together, these results demonstrate that compared to normal FET proteins, a larger fraction of FET oncoproteins bind, with an increased binding strength, to SWI/SNF.

Binding of normal and oncogenic FET proteins to SWI/SNF complexes could be expected to result in a competition for binding sites. To test this hypothesis, we expressed DsRED- or EGFP-tagged FUS-DDIT3, EWSR1-FLI1 or the tags alone in human HT1080 fibrosarcoma cells and quantified the normal and oncogenic FET proteins that co-precipitated with BRG1. Although the results show massive binding of the FET oncoproteins to BRG1 precipitates, no reduction of BRG1-bound FUS or EWSR1 proteins was observed (Fig 3C and D).

Based on our combined results, we propose alternative models for the binding between FET fusion oncoproteins and SWI/SNF chromatin remodeling complexes (Fig 3E). The divergent IP-binding/elution patterns between the normal and oncogenic FET proteins suggest that the fusion oncoproteins bind directly to SWI/SNF and not indirectly by multimerization with normal FET proteins (Fig 3E, models I and II). The lack of competition also suggests that normal and oncogenic FET proteins bind different variants of SWI/SNF or to different sites on the SWI/SNF complex (Fig 3E, models III and IV). As mentioned earlier, FET oncoproteins binds directly to normal FET proteins [31], and normal FET proteins form homo- and hetero-complexes. Furthermore, the many co-existing variants of SWI/SNF complexes [35,41] may have divergent binding properties. Several alternative complexes between FET proteins and SWI/SNF variants may thus co-exist in the tumor cells.

**SWI/SNF complexes bound by FET oncoproteins show normal core protein composition**

The composition of the SWI/SNF complex is changed by the SS18-SSX fusion oncoprotein in synovial sarcomas. The fusion protein
containing N-terminal parts of the SS18 SWI/SNF core protein evicts the BAF47 protein from the complex [42,43]. Even though FET proteins or their fusion partners are not considered core SWI/SNF constituents [35], they could theoretically cause a change in SWI/SNF composition. To test this possibility, we precipitated FUS-DDIT3 and EWSR1-FLI1 oncoproteins from MLS and EWS cell lines using DDIT3- and FLI1-specific antibodies. Since no normal DDIT3 or FLI1 is expressed in these cell lines, the antibodies only precipitate the fusion oncoproteins and their interacting proteins. MS analysis of the precipitates demonstrated complete sets of SWI/SNF core proteins co-precipitated with the fusion oncoproteins (Table 1 and Table EV3). These results rule out the possibility that oncogenic FET proteins cause loss of core proteins from SWI/SNF and indicate a different mechanism of action compared to the SS18-SSX fusion oncoprotein [42,43]. It also indicates that the FET oncoproteins bind several SWI/SNF variants as all the alternative SWI/SNF core proteins found in the cells were also precipitated with the FET oncoproteins.

**Forced expression of FET oncoproteins causes increased H3K27me3 levels**

The binding of FET oncoproteins to SWI/SNF prompted us to investigate whether this could lead to any functional effects. SWI/SNF has been reported to balance the suppressive H3K27 trimethylation (H3K27me3) caused by the PRC2 polycomb repressor complex [33,34]. Oncogenic mutations of SWI/SNF components have been reported to disturb this balance, leading to increased polycomb activity and H3K27me3 levels [34]. Elevated H3K27me3 was for example reported in cells lacking the SWI/SNF core BAF47/SMARCB1 protein [44]. Our immunoblot analysis of histone modifications in protein extracts from human HT1080 sarcoma cells

| Protein  | Alternative name | BRG1 Co-IP | BRM Co-IP | ARID1A | ARID1B | ARID2 | BAF170 | BAF155 | BAF60A | BAF60B | BAF60C | BAF57 | BAF47 | BAF53A | BAF53B | BAF45A | BAF45B | BAF45C | BAF45D | BAF180 | SS18 | FUS | EWSR1 | TAF15 | FUS-DDIT3 | EWSR1-FLI1 | EWSR1-ERG |
|----------|------------------|------------|-----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|-------|------|-----|-----|-----|-----|---------|--------|
| BRG1     | SMARCA4          | MS, IB     | MS, IB    |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BRM      | SMARCA2          | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| ARID1A   | BAF250A          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| ARID1B   | BAF250B          | -          | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| ARID2    | BAF200           | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF170   | SMARCC2          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF155   | SMARCC1          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF60A   | SMARCD1          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF60B   | SMARCD2          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF60C   | SMARCD3          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF57    | SMARCE1          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF47    | SMARCB1          | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF53A   | ACTL6A           | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF53B   | ACTL6B           | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF45A   | PHF10            | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF45B   | DPF1             | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF45C   | DPF3             | -          | -         |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF45D   | DPF2             | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| BAF180   | PBRM1            | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| SS18     | SYT              | -          | IB        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| FUS      | TLS              | MS         | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| EWSR1    | EWS              | MS, IB     |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| TAF15    | TAF2N            | -          | MS        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| FUS-DDIT3| TLS-CHOP         | -          | IB        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| EWSR1-FLI1|                | -          | IB        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |
| EWSR1-ERG|                 | MS         |          |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |       |      |    |    |    |    | TBD     |

*: no tryptic peptides detected, **: not applicable, (): no unique peptides detected.
Figure 3.
showed that forced expression of the oncogene **FUS-DDIT3** leads to significantly higher H3K27me3 levels (Fig 4A and B, and complete data set in Fig EV2A-I). **EWSR1-FLI1** transfections also lead to increased but more variable H3K27me3 levels. Additional experiments verified the fusion oncoprotein-induced trimethylation of H3K27 (Fig EV2C, D, H and I). Treatment of the cells with the EZH2 inhibitor tazemetostat dramatically reduced the H3K27me3 level proving that the quantitative Western blot assay was functional (Fig EV2E and J). H3K4me3 levels were largely unaffected, and effects on H3K27 acetylation varied but showed no consistent trend. We observed a small, not statistically significant, increase in EZH2 expression (Fig 4A and B) while no increased co-precipitation with BRG1 was seen (Fig 4C and D). A plausible explanation for these results is that binding of FET oncoprotein to SWI/SNF negatively impacts its PRC2-balancing function leading to increased PRC2/EZH2 activity. The observed effects of FET oncoproteins are thus similar to those caused by some oncogenic mutations in SWI/SNF core complex proteins. The increase in H3K27me3 levels may seem minor but could translate to changed regulation of hundreds of genes.

Our results are in parts at odds with recently published studies on EWS cell lines, reporting no effects on H3K27me3 levels but instead increased H3K27 acetylation levels at specific genomic sites [24,45]. The diverging results could be explained by the use of different experimental systems. The normal FLI1 protein is constitutively expressed in HT1080 cells used in our study (Fig EV2D) while this protein is absent in EWS cells [46]. The constitutive FLI1 expression may block EWSR1-FLI1 binding and the histone acetylation effects induced by this oncoprotein. Also, ChIP-seq-based assays used for the EWS-specific studies show genomic sites and distribution of histone modifications, but may fail to detect minor but wide spread changes in H3K27me3.

**FUS-DDIT3-altered gene expression patterns overlap with PRC2-regulated gene-sets**

To further investigate downstream effects of the FET oncoproteins **FUS-DDIT3** and **EWSR1-FLI1**, we performed RNA-seq to compare the gene expression patterns of FET-oncogene transfected and wild-type HT1080 cells (n = 3–4) (Fig 4C and source data). Lists of > 2-fold regulated genes were compared with the database of gene-sets changed after “chemical and genetic perturbations” (CGP, 3,433 gene-sets) using the Gene Set Enrichments Analysis (GSEA) tool. **FUS-DDIT3** downregulated genes overlapped significantly with gene-sets upregulated in cells after knock-out of the PRC2 component EZH2 (q-value 1.68 × 10⁻¹¹) or reconstitution of SWI/SNF component BAF57/SMARCE1 (q-value 2.37 × 10⁻¹¹). Deletion of NIPPI1 (nuclear inhibitor of protein phosphatase-1) leads to degradation of EZH2 expression [47]. In line with this observation, **FUS-DDIT3** downregulated genes overlapped significantly with genes upregulated after knockdown of NIPPI1 (q-value 2.9 × 10⁻²²), further underscoring the effect on PRC2 activity. These results are compatible with our hypothesis that FUS-DDIT3-induced downregulation of genes involves disruption of the SWI/SNF-PRC2 balance (Fig 4C). In contrast, **FUS-DDIT3** upregulated genes showed no overlap with any gene-set at comparably low significance.

**EWSR1-FLI1**regulated genes showed significant overlaps with gene-sets from conditions where **EWSR1-FLI1** expression was modified. Moreover, our upregulated genes overlapped with CGP gene-sets containing **EWSR1-FLI1** upregulated gene-sets and our downregulated genes matched with CGP-sets containing **EWSR1-FLI1** downregulated genes, indicating that the **EWSR1-FLI1** transfected HT1080 cells mimicked EWS cells (Fig 4C and source data). In contrast to the **FUS-DDIT3** expressing cells, there were no comparably low q-score overlaps between **EWSR1-FLI1** regulated gene lists and gene-sets from PRC2- or SWI/SNF-regulated conditions. This indicates divergent effects of **FUS-DDIT3** and **EWSR1-FLI1** in HT1080 cells even though SWI/SNF is the major binding partner of both oncoproteins.

The divergent effects between **FUS-DDIT3** and **EWSR1-FLI1** may be explained by the very different properties of the DDIT3 and FLI1 transcription factor partners. DDIT3 is a leucine zipper-containing, dimer-forming transcription factor of the CEBP family. It has, however, an acidic DNA-binding domain and is therefore considered to exert a dominant negative function, blocking DNA binding of most
Figure 4.
A unifying pathogenic mechanism for the FET family of fusion oncoproteins

The many variants of FET fusion oncoproteins are, with few exceptions, specific for one tumor type each (Fig 4D), and previous studies have shown that FET oncogenes are instructive for tumor morphology and gene expression patterns [25,53]. From Fig 4D, it is obvious that the transcription factor partners and their sequence specificity determine the tumor type. The recruitment of SWI/SNF to genomic sites determined by the FET oncoprotein was reported by Boulay et al [45]. Our results, showing that all FET-NTDs bind SWI/SNF, suggest that this aberrant SWI/SNF recruitment is a central mechanism behind the tumor type specificity and reflects a common pathogenic mechanism.

Malignant rhabdoid tumors and synovial sarcoma carry mutations in the BAF47/INI1/SNF5/SMARCB1 and SS18 SWI/SNF core components, respectively, that impair SWI/SNF functions. These tumors contain few additional mutations and are genetically stable [54,55], indicating the central role of SWI/SNF mutations in tumor development. Similarly, FET-oncogene-caused tumors are genetically stable with few other mutations and importantly, no reports of SWI/SNF mutations. Our data instead suggest that FET oncoproteins bind SWI/SNF and compromise its function. These common features of FET-oncogene-caused tumors, malignant rhabdoid tumors, and synovial sarcomas further underscore the importance of SWI/SNF and chromatin remodeling in cancer development and point to a novel unifying pathogenic mechanism for FET oncoprotein-associated tumors. Targeting of this mechanism may be a fruitful avenue for new therapies against all entities of this large group of tumors.

Materials and Methods

Cell culture

The Raji Burkitt lymphoma cell line [56] was a kind gift from Dr. Georg Klein, Karolinska Institutet. Myxoid liposarcoma (MLS) cell lines 402-91 and 2645-94 were established by us from MLS tumor tissues [57]. The fibrosarcoma cell line HT1080 [58] was obtained from ATCC (CCL-221, Manassas, VA, USA) and stable clones expressing FUS-DDIT3-EGFP, EWSR1-FLI1-EGFP, DDIT3-EGFP, and EGFP were established as described [59]. Possible mutations in genes encoding SWI/SNF components were ruled out by inspection of the COSMIC database and our own analysis on protein level. All genes encoding SWI/SNF components were ruled out by inspection of the COSMIC database and our own analysis on protein level. All genes encoding SWI/SNF components were ruled out by inspection of the COSMIC database and our own analysis on protein level.
Stable expression of EGFP constructs were maintained by addition of 500 μg/ml Genetecin. All media and supplements were obtained from Life Technologies (Carlsbad, CA, USA). Cells were maintained at 37°C with air containing 5% CO2. The unique fusion oncogene content of all used sarcoma cell lines was confirmed by RT–PCR analysis (Fig EV4).

An expression vector containing the full coding region of EWSR1-FLI1 (type 1) in pEGFP-N1 (Clontech, Mountain View, CA, USA) was made using the primers EWSR1xhoIF: ATACTCGAGATGGCGTCG
CAGGATATTACGTACC and FLISalIR: ATAGTCGGACCGGTAGTA
GCTGCGCTAAGTTGAAGG in the same way as described for FUS-DDIT3 [59]. Transient transfection of HT1080 cells with the pEGFP-N1 expression vector (empty or containing the fusion oncogenes FUS-DDIT3 or EWSR1-FLI1) or with pDsRED1-N1 (Clontech) expression vector (empty or with FUS-DDIT3, cloned same way as in pEGFP-N1) was done using FuGENE6® Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer’s recommendations. Cells were transfected the day after seeding, at 60% confluence with a transfection reagent (μl) to DNA (μg) ratio of 3:1. Nuclear extracts were made 24 h after transfection for dsRED/EGFP constructs, and whole-cell extracts were made 24 and 48 h after transfection for EGFP constructs to study histone modifications.

For PLA analysis, cells were grown on collagen I coated 8-well culture slides (BD Biosciences, San Jose, CA, USA). For tazemetostat treatment (EZH2-inhibition), cells were seeded on 6-wells plates and treated with 5 μM tazemetostat (EPZ-6438, Selleckchem, Munich, Germany) dissolved in DMSO, or DMSO-control, for 72 h before whole-cell extraction.

**Recombinant protein expression and pulldown**

Vectors encoding GST fusion proteins, recombinant oncogene expression, and purification were previously described [39]. Briefly, expression vectors were transformed to Rosetta DE3 pLysS (Novagen, Merck, Darmstadt, Germany) and inoculated in Luria broth (MP Biomedicals, Santa Ana, California, USA) with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol (both Sigma-Aldrich, St. Louis, MO, USA). Bacteria were grown overnight at 37°C with orbital shaking, diluted 1:20, and grown to an OD600 of 0.6 after which protein expression was induced by addition of IPTG (Merck) to a final concentration of 1 mM. After 4 h, bacteria were harvested by centrifugation at 6,000 g for 10 min at 4°C, frozen in liquid N2, and stored at −80°C. Pellets were thawed and resuspended in ice-cold lysis buffer (50 mM NaH2PO4 pH 7.5, 0.5% NP-40, 300 mM NaCl) supplemented with 5 mM DTT and protease inhibitors (Roche Diagnostics, Mannheim, Germany) followed by sonication. Samples were centrifuged at 12,000 g for 20 min at 4°C and the supernatants were incubated with pre-equilibrated Glutathione-Sepharose 4B (GE Healthcare) with bound GST fusion proteins was incubated with protein extracts for 2–4 h with rotation at 4°C. The sepharose was then washed four times with 1 ml protein extraction buffer. Immobilized protein complexes were eluted by denaturation at 95°C for 10 min in 2x LDS Sample Buffer (Invitrogen, Thermo Fisher Scientific). For experiments with varied NaCl concentration, the sepharose was washed four times with protein extraction buffer containing 100, 150, 250, 500, or 1,000 mM NaCl followed by one additional wash step with 100 mM NaCl to equalize the beads before elution. For experiments including nucleases, 20 U/ml RNase ONE Ribonuclease (Promega) or 10 U/ml RNase-Free DNase (Promega) were used in a modified protein extraction buffer with 5 mM MgCl2, 5 mM MnCl2, and 10 mM CaCl2. Samples were stored at −20°C. Pulldown samples were run on a gel (see SDS–PAGE and Immunoblot), and suitable gel pieces were sent for MS analysis (Study 1).

**In situ proximity ligation assays and confocal imaging**

Cells were washed briefly with PBS and fixed with 3.7% formaldehyde (Sigma-Aldrich) in PBS for 15 min. Samples were washed 3 × 10 min with PBS and incubated with block/permeabilization (B/P) buffer (PBS pH 7.3, 2% BSA, 0.02% Triton X-100; all from Sigma-Aldrich) for 30 min. Samples were then incubated for 90 min with combinations of primary antibodies: 1 μg/ml ARID1A (HPA005456; Sigma-Aldrich), 4 μg/ml BRG1 (H88) (sc-10768; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3 μg/ml DDIT3 (9C8) (ab11419; Abcam, Cambridge, United Kingdom), 2 μg/ml FUS (4H11) (sc-47711; Santa Cruz) diluted in B/P buffer. Control experiments were included by omitting one of two paired primary antibodies. Slides were washed 3 × 5 min in PBS with 0.1% Tween-20 with gentle agitation. In situ proximity ligation assay (PLA) was performed with Duolink In Situ Fluorescence products (Olink, Uppsala, Sweden) according to the manufacturer’s instructions. Briefly, samples were incubated for 1 h at 37°C with a mixture of PLA probes anti-rabbit PLUS and anti-mouse MINUS, each diluted 1:5 in B/P buffer. Slides were washed 2 × 5 min in 1x Wash Buffer A (Olink), and subsequent ligation, wash steps, and amplification with Duolink In Situ Detection Reagents Red (Olink) were prepared according to the manufacturer’s instructions. Slides were mounted with cover slips using Duolink In Situ Mounting Media with DAPI (Olink). A Zeiss LSM510 META confocal microscope system with LSM-5 software (Zeiss, Oberkochen, Germany) was used for confocal imaging. A 63x/1.4 oil objective and sequential scanning with excitation and META detector filter settings appropriate for each fluorophore was used (excitation 561 nm and BP600-710 for PLA signals, excitation 405 nm and BP420-475 for DAPI).

**Nuclear protein extraction and co-immunoprecipitation**

Cells from two confluent T75 or 15-cm petri dishes were harvested by scraping in PBS (Life Technologies) followed by centrifugation at
450 g for 10 min at 4°C. The cell pellet (approximately 100–200 µl packed cell volume) was resuspended in 5 packed cell volumes of hypotonic lysis buffer (10 mM KCl, 10 mM Tris pH 7.5, 1.5 mM MgCl2; all Life Technologies) supplemented with 1 mM DTT (Sigma-Aldrich) and 1x Halt Protease Inhibitor Cocktail (Thermo Scientific, Thermo Fisher Scientific) and was allowed to swell for 15 min on ice. The supernatant was discarded after centrifugation at 400 g for 5 min at 4°C. Packed cells were resuspended in 2 packed cell volumes hypotonic lysis buffer and disrupted by 2-5 strokes of a syringe with a 27-gauge needle. The cytoplasmic fraction was removed after centrifugation at 10,000 g for 20 min at 4°C. Pelleted nuclei were then resuspended in (2/3) packed cell volumes high-salt extraction buffer [0.42 M KCl, 10 mM Tris pH 7.5, 0.1 mM EDTA (all Life Technologies), 10% glycerol (Merck Chemicals, Merck)] supplemented with 1x Halt Protease Inhibitor Cocktail, and gently agitated in an icebox for 30 min. The nuclear fraction was collected after centrifugation at 20,000 g for 5 min at 4°C and diluted to 150 mM salt concentration.

The nuclear extract/antibody mixture was incubated overnight at 4°C. Peptides were extracted from the gel with 50% ACN in 1% acetic acid and dried down. Formic acid (Study 2) and LDS (Study 3 and 4) eluates from the immunoprecipitation (IP) were digested with trypsin using the filter-aided sample preparation (FASP) method [60]. Briefly, samples were reduced with 100 mM dithiothreitol at 60°C for 30 min, transferred to 30 kDa MWCO Pall Nanosep centrifugal filters (Sigma-Aldrich), washed with 8 M urea repeatedly, and alkylated with 10 mM methyl methane thiosulfonate. Digestion was performed in 50 mM TEAB, 1% sodium deoxycholate (SDC) buffer at 37°C by addition of 0.30 µg Pierce MS grade trypsin, and incubated overnight. An additional portion of trypsin was added and incubated for another 2 h. Peptides were collected by centrifugation, and SDC was removed by acidification with 10% trifluoroacetic acid. Samples were desalted using PepClean C18 spin columns (Thermo Fisher Scientific) according to the manufacturer’s guidelines and dried down. The sample in Study 4 was processed through a HiPPR detergent removal spin column (Thermo Fisher Scientific) prior to C18 desalting. Samples were reconstituted in 3% ACN in 0.2% formic acid (FA).

Peptide samples were analyzed on a hybrid linear ion trap-FTICR mass spectrometer equipped with a 7T ICR magnet (LTQ-FT, Study 1), an Orbitrap Fusion Trìbrid mass spectrometer (Study 2 and 3), or a Q Exactive HF (Study 4) mass spectrometer (all Thermo Fisher Scientific) interfaced with an Easy nLC 1000 liquid chromatography system. Peptides were trapped and separated using an C18 pre-column (45 × 0.075 mm I.D.) and analytical column (300 × 0.075 mm I.D.) packed with 3 µm Reprosil-Pur C18-AQ particles using a gradient from 5 to 80% ACN in 0.2% FA for 40 min. MS spectra and MS/MS spectra were acquired in the FTICR and the LTQ-trap, respectively. For each scan of FTICR, the three most intense double or triple charged ions were sequentially fragmented in the linear trap by collision-induced dissociation (CID). In study 2 and 3, precursor ion mass spectra were acquired at 120,000 resolution and MS/MS analysis was performed in a data-dependent mode where CID spectra of the most intense precursor ions were recorded in ion trap at 30,000 resolution and collision energy setting of 30 for 3 s (“top speed” setting). Charge states 2–7 were selected for fragmentation, dynamic exclusion was set to 45 s. In Study 4, the precursor ion mass spectra were acquired at 60,000 resolution and MS/MS analysis was performed in a data-dependent mode where HCD spectra of the Top 10 most intense precursor ions were recorded at 30,000 resolution. Charge states 2–4 were selected for fragmentation with collision energy setting of 28 and dynamic exclusion was set to 20 s.

Data analysis was performed utilizing Proteome Discoverer version 1.4 (Thermo Fisher Scientific) against the Human Swissprot Database version 55.3, 2009 (Study 1, verified against version Nov 2017 for data upload), May 2016 (Study 2), Sep 2016 (Study 3), and March 2017 (study 4). Mascot (2.3 or 2.5.1, Matrix Science) was used as a search engine with precursor mass tolerance of 5 ppm and fragment mass tolerance of 500 mnu. Tryptic peptides were

**Mass spectrometry**

Proteomic analyses were performed at The Proteomics Core Facility at the Sahlgrenska Academy, University of Gothenburg. Gel pieces (Study 1) were de-stained with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN), in-gel digested by addition of 10 ng/µl trypsin (Pierce MS grade, Thermo Fisher Scientific) in 50 mM ammonium bicarbonate, and incubated overnight at 37°C. Peptides were extracted from the gel with 50% ACN in 1% acetic acid and dried down. Formic acid (Study 2) and LDS (Study 3 and 4) eluates from the immunoprecipitation (IP) were digested with trypsin using the filter-aided sample preparation (FASP) method [60]. Briefly, samples were reduced with 100 mM dithiothreitol at 60°C for 30 min, transferred to 30 kDa MWCO Pall Nanosep centrifugal filters (Sigma-Aldrich), washed with 8 M urea repeatedly, and alkylated with 10 mM methyl methane thiosulfonate. Digestion was performed in 50 mM TEAB, 1% sodium deoxycholate (SDC) buffer at 37°C by addition of 0.30 µg Pierce MS grade trypsin, and incubated overnight. An additional portion of trypsin was added and incubated for another 2 h. Peptides were collected by centrifugation, and SDC was removed by acidification with 10% trifluoroacetic acid. Samples were desalted using PepClean C18 spin columns (Thermo Fisher Scientific) according to the manufacturer’s guidelines and dried down. The sample in Study 4 was processed through a HiPPR detergent removal spin column (Thermo Fisher Scientific) prior to C18 desalting. Samples were reconstituted in 3% ACN in 0.2% formic acid (FA).

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accepted with one missed cleavage, variable methionine oxidation and static cysteine propionamide modifications (Study 1) or zero to one missed cleavages and variable methionine oxidation, static cysteine methlythio modifications (Study 2, 3, and 4). The detected peptide threshold in the software was set to a significance level of Mascot 95% (Study 1) or Mascot 99% (Study 2, 3, and 4) by searching against a reversed database, and identified proteins were grouped by sharing the same sequences to minimize redundancy.

**Whole-cell extraction for histone modification analysis**

Whole-cell extracts were prepared on ice with cells from 70 to 95% confluent 10- or 15-cm petri dishes (stable clones) or 6-well plates (after transient transfection) by scraping in PBS followed by centrifugation at 450 g for 10 min at 4°C. The cell pellet was lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Pierce, Thermo Scientific) with 5 mM EDTA and 1x Halt Protease Inhibitor Cocktail and incubated on ice for 10 min with gentle mixing every 5 min. The lysate was then sonicated in order to disrupt viscous DNA. In order not to lose any histone proteins remaining in the insoluble fraction, no centrifugation was done at this point. Samples were then mixed with NuPAGE 4x LDS Sample Buffer to final concentrations of 106 mM Tris Base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM sodium deoxycholate, 0.1% SDS, Pierce, Thermo Scientific) with buffer (25 mM Tris–HCl pH 7.6, 50 mM NaCl, 2% glycerol, 0.1% SDS, 0.5% BSA, Life Technologies) according to the manufacturer’s instructions. In short, protein extracts were mixed with 1x NuPAGE system (Life Technologies) according to the manufacturer’s instructions. GraphPad Prism software (version 7.00, GraphPad, San Diego, CA, USA) was used for statistical analysis. The Student’s t-test (unpaired, two-sided t-test) was used with α < 0.05 considered significant. Original data for all quantifications are shown in the source data.

**SDS–PAGE and immunoblot**

Protein samples were size-separated with SDS–PAGE using the Novex NuPAGE system (Life Technologies) according to the manufacturer’s instructions. In short, protein extracts were mixed with 1x NuPAGE LDS sample buffer and 10% NuPAGE sample reducing agent, denatured at 70°C for 10 min, and separated on NuPAGE 4–12% Bis-Tris or 3–12% Tris-acetate gels. Separated proteins were stained with SimplyBlue SafeStain (Life Technologies) or transferred to polyvinylidene difluoride membranes (0.45 μm, Life Technologies) by wet blot. The membranes were blocked with 5% skim milk (Merck Chemicals) or 5% BSA (Sigma-Aldrich) in TBS-T buffer and 3 mM MgCl2, 5 mM dithiothreitol (both Invitrogen, Thermo Scientific), 1 M betaine (Sigma-Aldrich), 0.6 g/ml H3K4me3 (#05-745R; Merck Millipore), 0.1 g/ml H3K27me3 (#07-689; Merck Millipore), 0.1 g/ml EZH2 (#07-689; Merck Millipore), 0.5 g/ml BAF60A (E-6) (sc-135843; Santa Cruz Biotechnology, Inc.), 0.5 g/ml BAF 170 (E-6) (sc-135843; Santa Cruz Biotechnology, Inc.), 0.5 g/ml SS18 (H80) (sc-45766: eBioScience), 0.5 g/ml SS18 (sc-80), 0.5 g/ml BAF 170 (E-6) (sc-135843; Santa Cruz Biotechnology, Inc.), 0.5 g/ml BAF 170 (E-6) (sc-135843; Santa Cruz Biotechnology, Inc.), 0.5 g/ml FUS (4H11) (sc-47711; Santa Cruz Biotechnology, Inc.), 0.5 g/ml FUS-DDIT3-EGFP (sc-2745; Santa Cruz Biotechnology, Inc.), 0.5 g/ml GFP (JL-8) with 1x KAPA Hifi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA, USA), 0.1 μM primer (5’-AACGAGTGTTACCAACGGAGTACT30VN’-3’). Some membranes were stripped with ReBlot Plus (2504, Merck Millipore) during 15-min incubation in room temperature before relabeling the membrane with another primary antibody. Bands were quantified using Multi-Gauge V3.2 (Fujifilm, Tokyo, Japan).

**Statistical analysis**

Quantification values of immunoblots for the 3–4 replicates are presented as means ± SEM with each individual experiment indicated. The Student’s t-test (unpaired, two-sided t-test) was used with α < 0.05 considered significant. Original data for all quantifications are shown in the source data.

**RNA-sequencing**

The Smart-seq2 protocol [61] was used to generate sequencing libraries from HT1080 wt (n = 4), HT1080 EGF (n = 5), HT1080 FUS-DDIT3-EGFP (n = 3), and HT1080 EWSRI-FLI1-EGFP (n = 4). Adherent cells were washed with DPBS and scraped directly in RLT lysis buffer (Qiagen, Hilden, Germany) supplemented with β-mercaptoethanol (MP Biomedicals). Total RNA was extracted using the RNeasy Micro Kit with DNase treatment (Qiagen) according to the manufacturer’s recommendations and stored at −80°C. The RNA quality was confirmed using Agilent RNA 6000 Nano Kit on a 2100 BioAnalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA).

Reverse transcription was performed on 10 ng total RNA. An initial hybridization step was performed by adding 1 mM DNTP and 1 μM biotinylated adapter sequence-containing oligo-dT30VN (5’-biotin-AACGAGTGTTACCAACGGAGTACT30VN’-3’) to the sample (both Sigma-Aldrich, concentrations refer to the final reverse transcription reaction) and incubating at 72°C for 3 min. Subsequently, 1x first-strand buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, and 3 mM MgCl2), 5 mM dithiothreitol (both Invitrogen, Thermo Fisher Scientific), 10 mM MgCl2 (Ambion, Thermo Fisher Scientific), 1 M betaine (Sigma-Aldrich), 0.6 μM biotinylated adapter sequence-containing template switching oligonucleotide (5’-biotin-AACGAGTGTTACCAACGGAGTACT30VN’-3’). The RNA quality was confirmed using Agilent RNA 6000 Nano Kit on a 2100 BioAnalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA).

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amplification at 98°C for 20 s, 67°C for 15 s, and 72°C for 6 min, and a final additional incubation at 72°C for 5 min in a T100 instrument. Samples were transferred from 72°C directly to dry ice and stored at −20°C. Purification of samples was performed using Agencourt AMPure XP beads (BD Biosciences).

The 50 μl sample was mixed with 40 μl beads (beads-to-sample ratio of 0.8) followed by incubation at room temperature for 5 min on the bench and 5 min on a magnet (DynaMag, Thermo Fisher Scientific). Supernatant was discarded, and beads were washed twice with 200 μl 80% ethanol and left to dry. Elution of samples was performed with 17.5 μl RNase/DNase-free water (Invitrogen, Thermo Fisher Scientific) by incubation at room temperature for 2 min on the bench and 2 min on the magnet. Quality and concentration measurement was performed with Agilent High Sensitivity DNA Kit on a 2100 Bioanalyzer Instrument (Agilent Technologies), and 100 pg of cDNA was used for tagmentation and indexing.

Tagmentation and indexing were performed using Nextera XT DNA Library Preparation Kit and Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA). First, 10 μl Tagment DNA Buffer and 5 μl Amplicon Tagment Mix was added to 5 μl sample, and tagmentation was run at 55°C for 5 min in a T100 instrument. Next, 5 μl neutralize tagment buffer was added followed by centrifugation for 1 min at 1100 rpm (LMC-3000, rotor R-2, Biosoan, Riga, Latvia) and 5-min incubation at room temperature. For indexing and library amplification, 15 μl Nextera PCR Master Mix and 5 μl of each index 1 (i7) and index 2 (i5) adapters were added and amplification was run at 72°C for 3 min, 95°C for 30 s followed by 16 cycles of amplification at 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s, and a final additional incubation at 72°C for 5 min in a T100 instrument. Purification of samples was performed using Agencourt AMPure XP beads as before but using a beads-to-sample ratio of 0.6 by adding all sample volume to 30 μl beads.

The concentration of each sample was analyzed using Qubit dsDNA High Sensitivity Assay Kit (Invitrogen, Thermo Fisher Scientific). Library quality control and size was ensured by capillary gel-electrophoresis on a Fragment Analyzer using the DNF-474 High Sensitivity NGS kit (both Agilent technologies). Libraries were pooled equimolarly based on Fragment Analyzer data, and the final pool was quantified by qPCR using the NEBNext Library Quantification kit (New England BioLabs, Ipswich, MA, USA). The libraries were clustered at 1.8 pM supplemented with 1% PhiX control on a MiniSeq instrument (both Illumina) using paired-end sequencing with a read-length of 2 × 75 bp.

Alignment of Illumina reads was performed using STAR RNA-seq aligner v2.6 [62] using ENSEMBL GRCh38 assembly as the reference genome. Read count matrices were generated using the HTSeq python framework v0.9.1. [63]. Genes with a total count number < 10 were excluded from downstream analyses. Differential expression was analyzed using the R package DESeq2, based on shrink estimation for dispersion and fold-change using a negative binomial distribution model [64]. Adjusted P-values were calculated using the Benjamini–Hochberg method. Genes at least twofold regulated (adjusted P-value ≤ 0.05) were analyzed in downstream analysis using the molecular signature database (MSigDB) v6.2 [65,66]. Gene lists were compared to the gene-set collection “chemical and genetic perturbations” (GSEA: http://software.broadinstitute.org/gsea/msigdb/index.jsp), and top 20 gene-sets (FDR q-value < 0.05) for each comparison were selected.

Data availability

The mass spectrometry proteomics data from this publication have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications database (PRIDE) [67] [https://www.ebi.ac.uk/pride/archive/] and assigned the dataset identifier PXD012680. The RNA-sequencing data from this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) database [68] [https://www.ncbi.nlm.nih.gov/geo/] and assigned the identifier GSE125941.

Expanded View for this article is available online.

Acknowledgements

This study was supported by the Swedish Cancer Society (CAN2015/7130, 2016/438), The Swedish state under the agreement between the Swedish government and the county councils, the ALF agreement (ALFBG-722211 and 716321), Knut and Alice Wallenberg Foundation, Wallenberg Centre for molecular and translational medicine at University of Gothenburg, the Swedish Research Council (2017-01392), VINNOVA, the Swedish Society for Medical Research, the Assar Gabrielsons Foundation, the BioCARE National Strategic Research, the Swedish Childhood Cancer Foundation (PR2017-0043), the Johan Jansson Foundation for Cancer Research, the Swedish Society of Medicine, and the Wilhelm and Martina Lundgren Foundation for Scientific Research. We thank the Centre for Cellular Imaging at the Sahlgrenska Academy, University of Gothenburg, for help with imaging and Jacqueline Forzelius at #explainartist for graphical presentation. The Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University, performed the mass spectrometry analysis for protein identification. We are grateful of Inga-Britt and Arne Lundbergs Forskningsstiftelse for the donation of the Orbitrap Fusion Tribrid MS instrument.

Author contributions

ML and CT contributed with the main laboratory work, planned and designed experiments, prepared figures and other result presentations, and took a main part in writing the manuscript. PG made the histone analysis and associated cell culture/cloning work and wrote corresponding parts of the manuscript. EJ helped with cell culture work, data handling, and formatting and helped writing the manuscript. DA and SD contributed with manuscript writing and figure preparation; RR made important lab work and helped writing the manuscript. CV added the EZH2 analysis and helped writing the manuscript. MLS helped with bioinformatics analysis of RNA-seq data. HF and AS contributed with critical data interpretation and writing of the manuscript. PÅ contributed with project planning, experimental design, data handling and interpretation, and writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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