

A phosphorylation–deubiquitination circuit drives spatial reorganization of perinucleolar heterochromatin to restrain tumor immunogenicity

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A phosphorylation–deubiquitination circuit drives spatial reorganization of perinucleolar heterochromatin to restrain tumor immunogenicity

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Abstract

Subnuclear compartmentalization has emerged as a critical determinant of transcriptional homeostasis and cell-fate determination. The nucleolus scaffolds specialized heterochromatin, yet the mechanisms orchestrating its spatial assembly and therapeutic relevance remain elusive. Here we identify the nucleolar deubiquitinase OTUD4 as a key coordinator of heterochromatin architecture. We demonstrate that casein kinase II (CK2)-mediated phosphorylation serves as a molecular switch to activate OTUD4, which functions as a phospho-activated, K63-specific deubiquitinase. Crucially, we show that the spatial anchoring of H3K9me3-marked heterochromatin to the nucleolar surface is molecularly mandated by the K63-linked ubiquitination status of Nucleophosmin (NPM1) at the Lys27 (K27) residue. OTUD4-mediated deubiquitination of NPM1 K27 is indispensable for stabilizing the NPM1 pentameric scaffold, which in turn sequesters H3K9me3-marked domains at the nucleolar periphery. Disruption of the CK2–OTUD4–NPM1 axis triggers heterochromatin detachment, eliciting nucleolar stress and retroelement derepression. This epigenetic collapse activates cytosolic DNA-sensing pathways to potentiate tumor-intrinsic immunogenicity. Clinically, an *OTUD4*-nucleolus gene signature correlates with adverse survival and immunotherapy resistance. Together, these findings establish a phosphorylation–deubiquitination cascade to preserve heterochromatin topology and nominate spatial epigenetic targeting as a translational strategy to overcome immune evasion in refractory malignancies.

Keywords

Deubiquitination, Genome compartmentalization, Heterochromatin, Immunogenicity

98 INTRODUCTION

99 The eukaryotic genome is hierarchically organized into spatially and functionally
100 specialized nuclear compartments that orchestrate fundamental biological processes,
101 including transcription, DNA replication, stress adaptation, and lineage commitment¹.
102 This higher-order architecture transcends mere structural scaffolding; it enforces
103 topological homeostasis by segregating transcriptionally permissive euchromatin from
104 repressive heterochromatin, thereby sculpting the gene expression landscapes
105 requisite for cellular identity². While the maintenance of these subnuclear territories is
106 essential for physiological function, the molecular mechanisms governing the
107 assembly and stability of specific chromatin domains—particularly those sequestered
108 within specialized nuclear niches—remain incompletely understood.

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110 Heterochromatin serves as a fundamental structural and regulatory cornerstone of
111 subnuclear territories. This repressive state is classically bifurcated into two distinct
112 modalities: facultative heterochromatin, marked by H3K27me3 and H2AK119ub1,
113 which mediates developmental gene silencing, and constitutive heterochromatin,
114 enriched in H3K9me2/3 and H4K20me3, which preserves genome stability by
115 repressing transposable elements and repetitive DNA⁵. The topographical distribution
116 of these heterochromatin marks defines distinct nuclear compartments—H3K9me2-
117 enriched domains typically associate with the nuclear lamina, whereas H3K9me3-
118 marked heterochromatin localizes to the nuclear peripheral, perinucleolar and
119 pericentromeric regions^{6,7}. Although historically regarded as relatively inert structural
120 compartments, heterochromatin domains are now recognized as dynamic regulatory
121 hubs. Specifically, the spatial sequestration of repetitive elements within these domains
122 prevents their aberrant activation, which would otherwise trigger cytosolic nucleic acid-
123 sensing pathways and perturb cellular homeostasis^{8,9}. However, the mechanisms that
124 establish and maintain this intricate heterochromatin topology, as well as its broader
125 physiological implications, remain poorly defined.

126

127 Beyond its canonical function in ribosome biogenesis, the nucleolus has emerged as
128 a dynamic architectural and signaling nexus. Centered upon ribosomal DNA (rDNA)

arrays, nucleolar organization provides the scaffold for expansive chromatin domains that harbor silenced rDNA repeats, constitutive heterochromatin, and lineage-restricted gene clusters³. Through spatial sequestration of repressed genomic elements, nucleolus-associated heterochromatin facilitate nucleolar assembly, safeguard genomic integrity, and buffer transcriptional noise amidst cellular stress⁴. Increasing evidence suggests that nucleolar integrity is intimately linked to cellular adaptation and genomic fitness; however, the precise molecular determinants governing nucleolus-directed heterochromatin organization—and the functional consequences of its disruption—remain largely unexplored.

Here, we identify a nucleolar phosphorylation–deubiquitination cascade centered on OTUD4 that orchestrates perinucleolar heterochromatin organization and couples nuclear architecture to immune regulation. We demonstrate that phosphorylation by casein kinase II (CK2)-mediated phosphorylation activates the K63-specific catalytic competency of OTUD4, which specially deubiquitinates K63-linked chains from NPM1 at the Lys27 (K27) residue. This specific catalytic event stabilizes NPM1 oligomerization, thereby spatial anchoring H3K9me3-marked chromatin to the nucleolar surface. Disruption of the CK2–OTUD4–NPM1 axis detaches perinucleolar heterochromatin, provoking nucleolar stress and retroelements derepression, which activate cytosolic DNA-sensing pathways and enhance tumor-intrinsic immunogenicity. These findings uncover nucleolar heterochromatin organization as a previously unrecognized determinant of immune evasion and nominate its pharmacologic disruption as a strategy of spatial epigenetic therapy to overcome immunotherapy resistance.

RESULTS

OTUD4 depletion redistributes heterochromatin

To systematically identify nucleolar regulators of heterochromatin assembly, we performed a nucleolomics-targeted CRISPR–Cas9 loss-of-function screen, coupling fluorescence-activated cell sorting with quantitative assessment of H3K9me3 signal intensity, a defining feature of heterochromatin⁴ (Fig. 1a). Integrating parallel screens

conducted in B16F10 and YUMMER1.7 cells, we identified 29 shared negative regulators of H3K9me3, whose genetic ablation consistently increased global H3K9me3 levels (Fig. 1b; Supplementary information, Fig. S1e). Among the most significantly depleted sgRNAs were canonical heterochromatin organizers, including Cbx5 (encoding heterochromatin protein 1 α , HP1 α), Dnmt1, and Ddx27, thereby validating the specificity and robustness of the screening strategy^{10–12} (Fig. 1c; Supplementary information, Fig. S1a–e). In addition to shared hits, the screen revealed context-dependent regulators that scored preferentially in individual cell lines (Supplementary information, Fig. S1a–d). YUMMER1.7-specific hits included *Npm1* (encodes nucleophosmin 1, NPM1), *Myh9*, *Wdr74*, *Csnk2a1* (casein kinase 2 alpha 1, CK2 α 1) and *Rbx1* (a core E3 ubiquitin ligase component)¹³ (Supplementary information, Fig. S1a, b). B16F10-specific hits included *Trim28*, *Snrpa*, *Tbl3*, and *Csnk2a2* (CK2 α 2) (Supplementary information, Fig. S1c and d). As a canonical heterochromatin organizer, HP1 α is known to recruit H3K9me3-marked heterochromatin to NPM1—a nucleolar scaffold harboring a central histone-binding domain¹⁰—for stabilizing perinucleolar heterochromatin architecture¹⁴. Notably, among the top shared negative regulators identified across both screens were *Otud4*, encoding the ovarian tumor domain-containing deubiquitinase OTUD4, and its upstream kinase casein kinase 2 (CK2) (Fig. 1c; Supplementary information, Fig. S1a–e)¹⁵. OTUD4 was of particular interest, as it emerged as the sole deubiquitinase identified in the screen despite prior reports failing to detect its localization to the nucleolus. Moreover, its role in nucleolar homeostasis and heterochromatin organization has remained unexplored. These observations prompted us to investigate whether OTUD4 functions as a previously unrecognized regulator of heterochromatin assembly.

Perinucleolar heterochromatin is classically enriched for H3K9me3⁴. Consistent with our screening results, genetic ablation of *Otud4* led to a pronounced global increase in H3K9me3 levels, whereas peripheral heterochromatin marked by H3K9me2 remained largely unaffected (Fig. 1d; Supplementary information, Fig. S1f). Moreover, *Otud4* depletion specially resulted in the collapse of H3K9me3-enriched perinucleolar

chromatin, which became redistributed throughout the nucleoplasm and assembled into irregular foci across *OTUD4*-deficient cells (Fig. 1e; Supplementary information, Fig. S1g, h). Concordantly, the perinucleolar enrichment of HP1 α —a key mediator of H3K9me3-dependent heterochromatin tethering¹⁴—was disrupted, with HP1 α accumulating in aberrant nuclear foci in *Otud4*-deficient YUMMER1.7 cells (Fig. 1e). Electron microscopy (EM) images further disclosed a pronounced heterochromatin disengagement (Fig. 1f; Supplementary information, Fig. S1i). In addition, H4K20me3- and H3K27me3-marked heterochromatins have also been reported to localize to nucleolar-adjacent regions in certain contexts^{16,17}. In *Otud4*-deficient YUMMER1.7 cells, global levels of H4K20me3 and H3K27me3 were increased (Fig. 1d). In human RCC4 cells, *OTUD4* loss selectively elevated H4K20me3 without affecting H3K27me3 (Supplementary information, Fig. S1f). Notably, despite these global changes, neither H4K20me3- nor H3K27me3-enriched perinucleolar chromatin exhibited detachment or redistribution following *OTUD4* loss (Fig. 1e; Supplementary information, Fig. S1g, h). Previous studies also have shown that perinucleolar heterochromatin detachment is often coupled with increased histone acetylation, including H3K27ac¹⁸; accordingly, *OTUD4*-deficient cells displayed elevated global H3K27ac levels (Fig. 1d; Supplementary information, Fig. S1f). Collectively, these findings demonstrate that *OTUD4* is required to specially anchor H3K9me3-enriched heterochromatin at the nucleolar periphery, and that its loss precipitates spatial disengagement and nucleoplasmic redistribution of perinucleolar heterochromatin.

***OTUD4* depletion induces nucleolar stress**

Nucleolus-associated heterochromatin not only constitutes an integral component of nucleolar architecture but also serves as a structural platform for nucleolus reassemble during cell division¹⁹. EM images revealed a transition from disordered and irregular nucleoli to more compact and rounded nucleoli in *OTUD4*-deficient cells (Fig. 1f; Supplementary information, Fig. S1i). Immunofluorescence staining further corroborated *Otud4* depletion-induced morphological alterations in nucleoli, which adopted a more rounded and regular shape, delineated by a faint ring of NPM1 that marked a pronounced reduction in nucleolar motility (Fig. 1g; Supplementary

information, Fig. S1j). Furthermore, *Otud4* deficiency resulted in the accumulation of large FBL aggregates (a marker of the dense fibrillar component, DFC), together with RPA40 aggregates (a marker of the fibrillar center, FC), reflecting a global disorganization of the nucleolar structure (Fig. 1g; Supplementary information, Fig. S1j). While normal YUMMER1.7 cells typically exhibit 3–4 nucleoli per cell with relatively large dimensions (approximately 8–9 μm in diameter and an area of 40 μm^2), *Otud4*^{-/-} cells displayed a significant reduction in both nucleolar number and size (Fig. 1g). *OTUD4* depletion similarly affected the nucleolar morphology in the human lung cancer cell line A549 (Supplementary information, Fig. S1j). Together, these results establish OTUD4 as housekeeper of nucleolar architecture.

Given the structural perturbations, we asked whether OTUD4 also influences nucleolar function, including the synthesis and processing of rRNA and ribosome assembly. In the absence of *OTUD4*, the cells manifested defects in pre-rRNA processing and ribosome assembly (Fig. 1h-j; Supplementary information, Fig. S1k-m), paralleled by a remarkable decline in global protein translation (Fig. 1k; Supplementary information, Fig. S1n). It is widely recognized that structural and functional disruption of nucleoli causes "nucleolar stress," hence activating cellular surveillance mechanisms such as p53 signaling²⁰. Consistently, *Otud4*-deficient cells displayed an elevation in the abundance of p53 within the nucleoplasm and nucleoli (Fig. 1l; Supplementary information, Fig. S1o). Together, these data indicate that OTUD4 ablation provokes nucleolar stress.

To elucidate the structural determinants of OTUD4 requisite for maintaining perinucleolar heterochromatin architecture and nucleolar morphology, we initially interrogated its subcellular distribution. Although OTUD4 was predominantly cytoplasmic, it was detected within the nucleoplasm and nucleolus (Fig. 1m, n; Supplementary information, Fig. S1p, q). Structurally, OTUD4 comprises an N-terminal OTU domain (responsible for deubiquitinase (DUB) activity), an RNA-binding Tudor domain, and three predicted intrinsically disordered regions (IDRs)²¹ (Fig. 1o; Supplementary information, Fig. S1r). To define the essential domains of OTUD4

required for perinucleolar heterochromatin disengagement and nucleolar morphology, we reconstituted *Otud4*^{-/-} cells with multiple domain-deleted truncations. Notably, OTU- or IDR3-deleted truncations failed to restore the location of perinucleolar heterochromatin and nucleolar morphology (Fig. 1p; Supplementary information, Fig. S1s). Detailed amino acid sequence analysis revealed that IDR2 and IDR3 encompass a short arginine-rich linear motif (R-rich SLiM), which is critical for nucleolar localization²² (Fig. 1o). To further explore the function of the R-rich SLiM motif, we generated OTUD4 mutants by substituting three consecutive arginine (R) residues with alanine (A) residues. Only mutations in IDR3 but not in IDR2 abolished the function of full-length OTUD4; while intact OTUD4 or these chimeric truncations containing NoLS effectively rescued this conformation (Fig. 1p; Supplementary information, Fig. S1s). Strikingly, OTUD4 proteins harboring the IDR3 deletion or mutation were exclusively localized in the cytoplasm, whereas the chimeric OTUD4 truncations containing NoLS were primarily concentrated within the nucleolus (Supplementary information, Fig. S1t). Altogether, these results support that both nucleolar compartmentalization and catalytic DUB activity are indispensable for OTUD4-mediated orchestration of perinucleolar heterochromatin assembly and the maintenance of nucleolar integrity.

CK2-mediated phosphorylation of OTUD4 stabilizes NPM1 oligomerization to tether perinucleolar heterochromatin

To elucidate the role of OTUD4 in the perinucleolar heterochromatin coordination, we characterized its nuclear interactome. Cellular components (cytoplasm, nucleoplasm, and chromatin) were fractionated from HEK293T cells transfected with a Flag-vector control, full-length Flag-OTUD4, or the Flag-IDR3 mutant. Protein mass spectrometry of the nucleoplasmic fraction revealed that full-length Flag-OTUD4, but not the Flag-IDR3 mutant, robustly interacted with nucleolar proteins, such as NPM1, FBL, and nucleolin (NCL) (Fig. 2a; Supplementary information, Table S8). Additionally, OTUD4 was associated with chromatin modifiers, including TRIM28, which facilitates H3K9me3-marked heterochromatin assembly by recruiting histone methyltransferase SETDB1 and heterochromatin protein 1 (HP1)²³ (Fig. 2a). These interactions were further validated by co-immunoprecipitation (co-IP) assays conducted on whole-cell

and nucleolar fractions (Fig. 2b, c; Supplementary information, Fig. S2a-c). NPM1, which contains a central histone-binding region (amino acids 111–189), acts as a principal structural scaffold for perinucleolar heterochromatin¹⁰. Consistently, *OTUD4* deletion markedly reduced the interaction between NPM1 and the H3K9me3-marked heterochromatin complex (Fig. 2d). Thus, these findings identify OTUD4 as a key factor required for NPM1-anchored heterochromatin assembly at the nucleolar periphery.

Since OTUD4 harbors two R-rich SLiMs essential for binding the nucleolar protein NPM1 and ensuring its nucleolar localization²², we mapped the NPM1-binding domains of OTUD4. OTUD4 constructs with IDR3 depletion or mutation failed to interact with NPM1 (Supplementary information, Fig. S2e), indicating that OTUD4 binds to NPM1 through its C-terminal R-rich region. Considering DUB activity of OTUD4 is essential for perinucleolar heterochromatin sequestration, we hypothesized that OTUD4 may be involved in the deubiquitination of NPM1. Ubiquitin linkage analysis revealed that NPM1 undergoes both K48- and K63-linked ubiquitination (Supplementary information, Fig. S2d), with a pronounced increase in K63-linked ubiquitination in *OTUD4*-deficient cells (Fig. 2e, f). Cell-free deubiquitination assays verified that OTUD4 directly removed K63-linked ubiquitin chains from NPM1 (Fig. 2g). These findings demonstrate that OTUD4 remove K63-linked ubiquitin chains from NPM1.

To explore the structural basis of the OTUD4–NPM1 interaction, we analyzed OTUD4 binding to a series of NPM1 truncations. Structurally, NPM1 comprises the N-terminal oligomerization domain (OD), central acidic (AD), heterodimerization domain (HD) and C-terminal nucleic acid-binding domain (ND) (Supplementary information, Fig. S2f). Truncations containing OD retained the ability to bind wild-type Flag-tagged OTUD4 (Supplementary information, Fig. S2g), indicating that OTUD4 engages NPM1 through its oligomerization domain. Because the N-terminal OD of NPM1 is essential for pentamerization, a structural feature essential for its biological functions²², we assessed the impact of *OTUD4* loss on NPM1 oligomerization by native

polyacrylamide gel electrophoresis (PAGE). In wild-type cells, NPM1 predominantly formed pentamers (~150 kDa), whereas in *Otud4*-deficient cells, monomeric NPM1 (~30 kDa) accumulated as the most abundant species (Fig. 2h). Thus, OTUD4 is indispensable for maintaining NPM1 oligomerization. We next sought to pinpoint the ubiquitination site on NPM1 that mediates this regulation. Lysine residues (K27, K32, K54, K80) within the OD were individually substituted with arginine (Supplementary information, Fig. S2f), and ubiquitination assays revealed that substitution of K27 with arginine (K27R) completely abrogated OTUD4 deficiency-induced K63-linked ubiquitination (Fig. 2i). Notably, overexpression of the K27R mutant restored NPM1 pentamer formation (Fig. 2j) and reversed the H3K9me3 perinucleolar segregation and the nucleolar shrinkage observed in *Otud4*-deficient cells (Fig. 2k). These results collectively establish that OTUD4 maintains NPM1 oligomerization through K63-linked deubiquitination at lysine 27, a modification that is critical for anchoring perinucleolar heterochromatin and preserving nucleolar morphology.

OTUD4 exhibits both K48- and K63-linked DUB activity and contains two phosphorylation sites, Ser202 and Ser204, within the conserved casein kinase II (CK2) recognition motif (SXXE/D) that shift its activity from K48- to K63-specific forms¹⁵. To dissect these activities, we generated catalytically inactive mutants by substituting the catalytic cysteine residue with alanine (C45A), a modification that disrupts OTUD4's K48-linked deubiquitinating activity while exerting minimal impact on its K63-linked DUB function. We also mutated two serine (S) residues to alanine (A) (S202/204A), which completely abolished the K63-linked DUB activity, whereas their substitution with aspartate (S202/204D) enhanced the K63-linked DUB activity (Supplementary information, Fig. S1h). In *Otud4*-deficient cells, overexpression of WT, C45A, or phosphomimetic S202/204D OTUD4—but not phosphorylation-deficient S202/204A mutants—reduced NPM1 K63-linked deubiquitination (Fig. 2l), diminished the levels of H3K9me3 (Supplementary information, Fig. S2i), re-established the interaction among OTUD4, NPM1, and heterochromatin components (Supplementary information, Fig. S2j). Conversely, pharmacological inhibition of CK2 by CX-4945 increased K63-linked ubiquitination of NPM1 (Fig. 2m), and disrupted the OTUD4–NPM1–heterochromatin

complex (Fig. 2n). In *Otud4*-deficient cells, overexpression of the S202/204D mutant effectively counteracted the effects of CK2 inhibitor (CX-4945) on NPM1 shape and H3K9me3 perinucleolar segregation (Fig. 2o). Indeed, CX-4945 disrupted nucleolar architecture, in thus far that it caused a marked reduction in the number and size of nucleoli, as it suppressed pre-rRNA processing and ribosome assembly (Fig. 2p-t; Supplementary information, Fig. S2k-n). These findings demonstrate that CK2-dependent phosphorylation activates OTUD4, enabling it to remove K63-linked ubiquitin chains from NPM1 and sustain perinucleolar heterochromatin organization (Fig. 2Aa).

To further examined whether the OTUD4–NPM1 interaction governs perinucleolar heterochromatin assembly, we engineered OTUD4-interfering peptide (OIP) that mimics the R-rich motif within IDR3 of OTUD4 and competitively bind to NPM1 N-terminal oligomerization domain (OD) by fusing them with a TAT membrane-penetrating peptide²⁴ (Fig. 2u). OIP treatment effectively blocked the interaction between OTUD4 and NPM1 (Fig. 2v; Supplementary information, Fig. S2o). Functionally, OIP treatment effectively induced segregation of H3K9me3-marked heterochromatin from the nucleolus (Fig. 2w; Supplementary information, Fig. S2p), and triggered hallmarks of nucleolar stress, as indicated by nucleolar disintegration, a marked reduction in nucleolar number and size, and suppression of pre-rRNA processing (Fig. 2x-z; Supplementary information, Fig. S2q-s). Collectively, these results demonstrate that the OTUD4–NPM1 interaction is critical for indispensable for perinucleolar H3K9me3 assembly and nucleolar homeostasis (Fig. 2Aa).

OTUD4 ablation derepresses heterochromatin-marked retroelements

Heterochromatin predominantly enriched repetitive elements, and the spatial remodeling of heterochromatin may entail the transcription of retrotransposons (RE, also referred to as retroelements)^{8,9,25}. We postulate that the OTUD4 depletion might compel perinucleolar heterochromatin disengagement to reactivate retroelements. Indeed, *Otud4*-deficient YUMMER1.7 exhibited transcriptional reactivation of several RE types, including long terminal repeat (LTR, such as RMER19A and MER21C) and

non-LTR elements like long interspersed nuclear elements (LINE, e.g., L1MEc and L1MB3), as determined by RNA sequencing (RNA-seq) (Fig. 3a-c). Quantitative real time polymerase chain reactions (qPCR) confirmed RE reactivation by *OTUD4* inhibition in both human and murine cancer cell lines (Supplementary information, Fig. S3a, b). Similarly, in patient-derived primary breast cancer organoids, *OTUD4* ablation resulted in upregulation of RE expressions, accompanied by mildly impairment of organoid formation and spheroidization (Fig. 3d-f). Collectively, these results demonstrate that OTUD4 loss derepress RE transcription.

To elucidate the mechanism underlying RE derepression upon OTUD4 loss, we first considered DNA methylation, a key repressive modification often colocalized with H3K9me3-enriched heterochromatin and essential for silencing RE¹¹. We performed whole-genome bisulfite sequencing (WGBS) to assess methylation status across the genome. The findings indicated no significant alterations of DNA methylation, either globally or within H3K9me3-marked regions, between wild-type and *Otud4*-depleted cells (Fig. 3c; Supplementary information, Fig. S3c, d). These results indicated that DNA methylation remains largely unaltered following *OTUD4* depletion and is therefore unlikely to account for RE activation.

Because chromatin modifications are also central to RE transcriptional control²⁶, we next examined whether OTUD4 loss alters histone modification patterns associated with perinucleolar heterochromatin. Previous studies have shown that NPM1 oligomerization stabilizes histone interactions^{14,27,28}, whereas its depolymerization destabilizes perinucleolar H3K9me3-marked heterochromatin¹⁴ and is often coupled with increased histone acetylation, including H3K27ac¹⁸. Consistent with this, our aforementioned imaging analyses revealed a re-distribution of perinucleolar H3K9me3 and an increase of H3K27ac level upon *OTUD4* depletion (Fig. 1d, e; Supplementary information, Fig. S1f-1h). To comprehensively assess these changes, we performed nucleolus-associated domain sequencing (NAD-seq), assay for transposase-accessible chromatin using sequencing (ATAC-seq), and H3K9me3 or H3K27ac-targeted cleavage under Targets and tagmentation sequencing (CUT&Tag-seq) in both

wild-type and *Otud4*-depleted cells. Notably, many nucleolus-associated domains (NADs) including diverse repetitive elements and RE, were reduced in *Otud4*-deficient cells. Furthermore, although global chromatin accessibility was reduced in *Otud4*-deficient cells, specific retroelement loci located in NADs exhibited increased accessibility, suggesting localized chromatin opening (Fig. 3c and g; Supplementary information, Fig. S3d, e). This was accompanied by a substantial reduction of RE at H3K9me3-marked regions (Fig. 3c, g; Supplementary information, Fig. S3d). Conversely, the majority of H3K27ac-marked regions showed enrichment following *Otud4* knockout, resembling active enhancer elements (Fig. 3c, g; Supplementary information, Fig. S3d). The upregulation of chromatin-opening RE sites strongly correlated with a reduction in NADs and H3K9me3 modifications, and a concurrent H3K27ac enrichment in *Otud4*-depleted cells, underscoring a shift from repressive to active chromatin (Fig. 3h). To explore the transcriptional regulators driving RE reactivation, we performed motif enrichment analysis of RE insertions that gained accessibility upon OTUD4 loss, revealing enrichment of interferon-stimulated response elements (ISRE) and motifs recognized by interferon regulatory factors (IRF) (Fig. 3i, j). Functionally, CRISPR-mediated deletion of *Irf1* in *Otud4*-ablated YUMMER1.7 cells substantially attenuated RE upregulation (Fig. 3k). These findings suggest that OTUD4 derepresses RE expression by fostering chromatin accessibility and guiding IRFs to RE loci, thereby initiating their transcriptional activation.

RE reactivation is frequently observed following the loss of H3K9me3 methyltransferase complexes, such as TRIM28 and SETDB1, which are required for heterochromatin formation and transposable element silencing^{8,23}. To test whether these complexes act downstream of OTUD4, we ablated *Trim28* or *Setdb1* in *Otud4*-deficient YUMMER1.7 cells. Notably, neither knockout further increased RE expression (Supplementary information, Fig. S3f, g), suggesting that these enzymes function within the same regulatory pathway. These results support a model in which OTUD4 preserves perinucleolar heterochromatin by stabilizing nucleolar architecture. Upon OTUD4 loss, H3K9me3-marked chromatin redistributes into the nucleoplasm, thereby facilitating RE activation (Fig. 3l).

439

440 **Retroelements driven by OTUD4 depletion potentiates DNA sensor-mediated**
441 **immunogenicity**

442 RE generate double-stranded RNA (dsRNA) by bidirectional transcription and dsDNA
443 via reverse transcription (RT), and both dsRNA and dsDNA accumulate in the cytosol
444 to activate various nucleic acid sensors to activate the MDA5/RIG-I/MAVS and
445 cGAS/STING pathways²⁹. These processes initiate pattern recognition receptor
446 signaling, triggering type I interferon (IFN) responses and stimulating MHC-I (major
447 histocompatibility complex class I, known as HLAs in humans) expression²⁹.
448 Consistently, genes upregulated in *Otud4*-depleted cells were markedly enriched for
449 genes involved in immune-related processes, including viral responses, type I IFN
450 responses, and antigen presentation as determined by RNA-seq (Fig. 4a), and
451 confirmed by RT-PCR in both human and murine cancer cell lines (Fig. 4b;
452 Supplementary information, Fig. S4a-b). Similarly, in patient-derived primary breast
453 cancer organoids, *OTUD4* deletion robustly induced the expression of interferon-
454 stimulated genes (ISGs), and HLA AP genes (Fig. 4c). In line with this interpretation,
455 *Otud4* ablation enhanced the protein expression of core MHC-I/HLA AP components
456 across multiple mouse and human cancer cell lines (Fig. 4d; Supplementary
457 information, Fig. S4c-f). Additionally, ex vivo analysis of *Otud4* knockdown tumors
458 demonstrated elevated MHC-I surface expression (Fig. 4e), confirming the negative
459 impact of OTUD4 on MHC-I in multiple experimental systems. Since MHC-I is a pivotal
460 regulator of CD8⁺ T cell activation and function, we investigated whether *Otud4*
461 deficiency potentiates antigen-specific CD8⁺ T cell responses. Notably, *Otud4*
462 deficiency enhanced cell surface expression of antigen-specific pMHC-I in
463 YUMMER1.7-Cas9 cells expressing ovalbumin (OVA) (Fig. 4f). These cells expressing
464 OVA stimulated interleukin-2 (IL-2) secretion by H-2K^b: OVA peptide-specific B3Z CD8⁺
465 T cell hybridoma cells. As compared to *Otud4*-expressing controls, *Otud4*-knockout
466 cancer cells stimulated enhanced IL-2 production by T cells (Fig. 4g). Consistently,
467 *Otud4*-deficient cells exhibited heightened susceptibility to lysis by OT-1 T cells, which
468 express a transgenic T cell receptor recognizing the OVA peptide presented by H-2Kb
469 MHC-I (Fig. 4h). Together, these results indicate that OTUD4 loss stimulate IFN and

antigen-presentation pathways, enabling tumor cells to evade T lymphocyte-mediated immune attack.

Next, we identified the dominant nucleic acid sensing pathway responsible for this response. Gene set enrichment analysis (GSEA) revealed an upregulation of cytosolic DNA-sensing and RIG-I-like receptor signaling in *Otud4*^{-/-} cells (Fig. 4i, j). To dissect the contribution of each pathway, we genetically ablated key components and found that *Sting* depletion, but not *Mavs* knockout, diminished the transactivation of ISGs and MHC-I/HLA AP genes, but without affecting RE expression in *Otud4*^{-/-} cells (Fig. 4k; Supplementary information, Fig. S4g). Similarly, in HEK293T cells—lacking endogenous cGAS³⁰—OTUD4 depletion led to elevated RE expression but failed to induce ISGs and HLA AP genes (Supplementary information, Fig. S4h). In vivo, knockout of *Sting*, but not of *Mavs*, rendered tumors resistant to *Otud4* deficiency-induced regression (Fig. 4l). Accordingly, OTUD4-deficient cancer cells exhibited accumulation of cytosolic dsDNA (Fig. 4m; Supplementary information, Fig. S4i), accompanied by the activating phosphorylation of STING (S366), TBK1 (S172) and IRF3 (S396) —confirming activation of the cGAS/STING pathway (Fig. 4n; Supplementary information, Fig. S4j). Treatment with the RT inhibitors (RTi), specifically the combination of zidovudine and nevirapine, decreased RE-derived cytosolic dsDNA levels and partially suppressed cGAS/STING signaling in *Otud4*^{-/-} cells (Fig. 4o, p). Transfection of cytosolic dsDNA isolated from *Otud4*^{-/-} cells into WT cells induced transcription of ISGs and MHC-I AP genes (Fig. 4q), reinforcing the functional relevance of RE-derived dsDNA. These findings suggest that *Otud4* depletion activates ISGs and MHC-I AP genes through the STING-dependent dsDNA sensing pathway, driven by the accumulation of RE-derived cytosolic dsDNA.

To further investigate the role of RE derepression in the OTUD4-deficiency phenotype, we modulated RE expression via epigenetic and CRISPR-based approaches. In accord with the notion that DNA methylation suppresses RE expression, and that tet methylcytosine dioxygenase 2 (TET2) knockout mimics RE hypermethylation³¹, we found that *Tet2* loss significantly reduced ISG, MHC-I/HLA and RE transcript levels in

Otud4-ablated cells (Supplementary information, Fig. S4k, l), while partially reversing the tumor-suppressive effect of *Otud4* deficiency (Supplementary information, Fig. S4m). To directly interrogate the impact of RE activation, we employed a CRISPR-dCas9-based transcriptional activation (CRISPRa) system with sgRNAs targeting the *L1MEC* promoter regions. Activation of *L1MEC* was confirmed by RT-PCR and led to upregulation of ISGs and MHC-I AP genes, along with increased MHC-I surface expression (Supplementary information, Fig. S4n, p). Conversely, CRISPR interference (CRISPRi) using dCas9-KRAB to repress endogenous *L1MEC* in *Otud4*-deficient cells reduced ISG and MHC-I genes expression and diminished surface MHC-I levels (Supplementary information, Fig. S4o, p). These results demonstrate that RE reactivation is both necessary and sufficient to drive cGAS/STING-dependent IFN signaling and MHC-I upregulation in the context of OTUD4 loss.

OTUD4 loss retards tumor growth through adaptive immunity

Next, we evaluated the in vivo effects of *Otud4* on a variety of mouse tumor models. To minimize Cas9 immunogenicity, we employed lentiviral-based short hairpin RNA (shRNA) for OTUD4 knockdown rather than CRISPR-Cas9 editing. *Otud4* knockdown in YUMMER1.7 cells delayed tumor growth and occasionally caused complete tumor regression (Supplementary information, Fig. S5a). Consistent reductions in tumor growth and even regressions were observed for other *Otud4*-deficient cancer cell lines compared to controls, including B16F10 melanomas, CT2A gliomas, MC38 colorectal carcinomas, MCA205 fibrosarcomas and LLC lung carcinoma (Fig. 5a; Supplementary information, Fig. S5b-g). Mice that successfully eliminated *Otud4*-deficient tumors exhibited protection against rechallenge with homotypic cells, indicating the establishment of durable immune memory (Fig. 5a; Supplementary information, Fig. S5c). Notably, *Otud4* depletion failed to affect cell growth in vitro or tumor growth in athymic hosts, as well as in mice lacking the common type I IFN receptor *Ifnar1* (Fig. 5b-d; Supplementary information, Fig. S5h). To confirm that the anti-tumor effect of *Otud4* ablation is linked to increased MHC-I expression, we abolished MHC-I surface expression in *Otud4*-KO tumour cells by depleting *B2m*. Remarkably, the suppression of MHC-I surface expression in *Otud4*-KO cancer cells abrogated the antineoplastic

function of *Otud4* deletion in tumour growth (Fig. 5e; Supplementary information, Fig. S5i). Thus, the antitumor effect of OTUD4 elimination fully depend on an active antitumor immune response.

YUMMER1.7 cells, derived from YUMM1.7 through ultraviolet B radiation, harbour somatic mutations that may generate neoantigens³². To assess the immunogenic potential of *Otud4*-depleted cells, we re-inoculated control or *Otud4*^{-/-} YUMM1.7 cells into mice previously engrafted with either control or *Otud4*^{-/-} YUMMER1.7 tumours. While control YUMM1.7 cells exhibited only a slight growth reduction in pre-exposed *Otud4*^{-/-} YUMMER1.7 hosts compared to control hosts, *Otud4*^{-/-} YUMM1.7 tumours were eradicated (Fig. 5f). Consistent findings were obtained with MCA205 fibrosarcoma (Supplementary information, Fig. S5j). Altogether these findings suggest that the tumour antigens responsible for rejection were shared among *Otud4*^{-/-} cells and specifically attributable to the loss of OTUD4.

OTUD4 loss inflames tumor-immune landscape

To explore the impact of OTUD4 ablation on the tumour-immune microenvironment (TIME) by performing single-cell RNA sequencing (scRNA-seq) of CD45⁺ leukocytes isolated from control and *Otud4*^{-/-} YUMMER1.7 tumours (Fig. 5g). Immune cell composition varied markedly between models, with *Otud4*^{-/-} YUMMER1.7 tumours showing a notable enrichment of myeloid cells, including CD86⁺ macrophage (Mφ) and conventional dendritic cells type-2 (cDC2)—key players in antigen presentation and T cell priming (Fig. 5h, i). Although the ratio of CD8⁺ and CD4⁺ T cell populations were comparable between control and *Otud4*^{-/-} YUMMER1.7 tumours, differential gene expression analysis revealed that CD8⁺ and CD4⁺ T cells in *Otud4*^{-/-} tumours downregulated exhaustion markers (e.g., *Lag3*, *Pdcd1* and *Havcr2*), while elevating activation markers such as *Cd44* (Fig. 5j). Flow cytometry confirmed these observations, showing enhanced activation and maturation of macrophages and dendritic cells in *Otud4*^{-/-} tumours, evidenced by the elevated levels of MHC-II and CD86 co-stimulatory molecules. Furthermore, OTUD4-ablated tumours exhibited enhanced infiltration of CD8⁺ T cell, accompanied by elevated expression of granzyme

B (GZMB), interferon γ (IFN γ), and the proliferation marker Ki-67 (Fig. 5k). Notably, these tumours also showed augmented infiltration of activated CD4⁺ T cells characterized by elevated IFN γ and Ki-67 expression, whereas regulatory T cell (Treg) frequencies remained unchanged across models (Fig. 5k). Immunohistochemistry (IHC) confirmed that *Otud4* loss significantly increased infiltration by CD8⁺ T cells, F4/80⁺ M ϕ , and CD86⁺ cells, but minimally increased CD4⁺ T cells in the tumour bed (Fig. 5l).

To evaluate the contribution of M ϕ and CD8⁺ T to antitumor effect of *Otud4* deficiency, we depleted M ϕ and CD8⁺ T cells in tumour-bearing mice using clodronate liposomes and anti-CD8 antibodies, respectively. Depletion of either M ϕ or T cells attenuated the tumour-suppressive effects of *Otud4* ablation (Fig. 5m; Supplementary information, Fig. S5k, l). Considering that increased MHC-I expression and the intratumoral presence of anti-tumour immune cells may bolster immunosurveillance, we explored whether this strategy can be potentiated by immune checkpoint blockade (ICB). Indeed, ablation of *Otud4* sensitized LLC tumors to treatment with a monoclonal antibody targeting PD-1 (Fig. 5n). These findings suggest that OTUD4 loss reshapes tumor-immune microenvironment, ultimately improving ICB efficacy.

CK2-OTUD4-NPM1 axis governs immunogenicity

Tumor-intrinsic immunogenicity represents the foundational priming event in the cancer-immunity cycle. This process is defined by two requisite facets: (i) antigenicity, reflecting the MHC-restricted presentation of antigens that distinguish malignant cells from their healthy counterparts, and (ii) adjuvanticity, driven by the emission of damage-associated molecular patterns (DAMPs) such as type I interferons that orchestrate adaptive immune mobilization^{33,34}. To delineate the structural requirements of OTUD4 for the suppression of tumor immunogenicity, we reconstituted *Otud4*^{-/-} cells with multiple OTUD4 domain-deleted truncations. Notably, OTU- or IDR3-deleted truncations were insufficient to suppress the aberrant upregulation of ISGs, MHC-I AP genes, RE, and cell-surface pMHC-I levels (Supplementary information, Fig. S6a-c). Given that short arginine-rich linear motif (R-rich SLiM) in IDR3 is critical for its

nucleolar localization. we generated OTUD4 mutants by substituting three consecutive arginine (R) with alanine (A). Mutational analysis revealed that these substitutions specifically within IDR3, but not IDR2, compromised the repressive capacity of OTUD4 (Supplementary information, Fig. S6a-c). To further corroborate whether the nucleolar localization and DUB activity of OTUD4 are required for the inhibition of tumor immunogenicity, we engineered chimeric OTUD4 truncations (OTU+Tudor or Δ R-rich SLiM) as well as full-length OTUD3 equipped with a classical nucleolar localization signal (NoLS). These chimeras effectively restored the repression of ISGs, REs, and MHC-I AP genes, while normalizing cell-surface pMHC-I levels in *Otud4*^{-/-} cells (Supplementary information, Fig. S6a-c). Of note, overexpression of OTUD4 constructs with the IDR3 deletion or mutation failed to rescue the anti-tumour effects observed upon *Otud4* loss in the mouse model (Supplementary information, Fig. S6d). Altogether, these results support the interpretation that OTUD4-mediated repression of tumor immunogenicity relies on both its DUB activity and nucleolar localization.

To determine which deubiquitination mode underlies this function, we dissected the contributions of OTUD4's K48- versus K63-linked DUB activity. Substitution of the catalytic cysteine residue with alanine (C45A) selectively impaired K48-linked activity while exerting minimal impact on its K63-linked DUB function. In parallel, mutation of serine residues S202 and S204 to alanine (S202/204A) abolished K63-linked activity, whereas their substitution with aspartate (S202/204D) enhanced it. Upon transfection into cells, C45A and S202/204D mutants (but not the control S202/204A mutant), effectively reduced the expression of ISGs, MHC-I AP genes, and RE, as well as the surface levels of MHC-I in *Otud4*^{-/-} YUMMER1.7 cells (Supplementary information, Fig. S6a-c). Consistently, transfection with C45A and S202/204D mutants completely rescued the growth defect of *Otud4*^{-/-} YUMMER1.7 tumours in incompetent mice (Supplementary information, Fig. S6d). Prior study has implicated CK2 in OTUD4 phosphorylation at S202 and S204¹⁵. Overexpression of the S202/204D mutant effectively attenuated the induction of ISGs, MHC-I AP genes, RE, and surface pMHC-I expression triggered by CK2 inhibition (Supplementary information, Fig. S6e, f). These results identify CK2-dependent phosphorylation of OTUD4 at S202/S204 as a

pivotal post-translational switch controlling its catalytic specificity and immunomodulatory capacity.

We next examined whether this OTUD4-NPM1 deubiquitination axis directly governs MHC-I expression. Both pharmacological inhibition of NPM1 oligomerization using NSC348884 and genetic silencing of *NPM1* markedly induced the expression of ISGs, MHC-I/HLA AP genes, and RE, ultimately enhancing MHC-I/HLA surface expression in both human and mouse cancer cells (Supplementary information, Fig. S6g-l). Conversely, ectopic expression of the NPM1 K27R mutant, which resists K63-linked ubiquitination, attenuated these transcriptional and phenotypic effects in *Otud4*-deficient cells (Supplementary information, Fig. S6n-m). Similarly, treatment with OTUD4-interfering peptide (OIP) induced the expression of MHC-I/HLA AP genes, concomitant with enhanced surface expression of MHC-I/HLA molecules (Supplementary information, Fig. S6o-t). Although previous studies have implicated OTUD4 in NF- κ B signalling¹⁵, a pathway known to regulate MHC-I transcription³⁵, we observed minimal differences in p65 expression or nuclear translocation between wild-type and *Otud4*^{-/-} cells both at baseline and following TNF α stimulation (Supplementary information, Fig. S6u, v). Collectively, these findings reveal that OTUD4 represses MHC-I expression through K63-specific deubiquitination of NPM1 rather than canonical NF- κ B signalling.

Targeting CK2-OTUD4-NPM1 axis augments immunogenicity and improve ICB response

We further evaluated the translational relevance of our findings. Anticancer drugs that induce nucleolar stress are particularly strong inducers of immunogenicity. Thus, a previous phenotypic screen performed on 1,180 anticancer agents, identified 152 compounds, including DNA intercalators (doxorubicin, epirubicin, and mitoxantrone), as effective inducers of nucleolar condensation³⁶. Plotting the nucleolar normality score (NNS) against the calculated immunogenic cell death (ICD) score³⁷ confirmed that such DNA intercalators have the potential to induce ICD and unravelled CX-4945, a selective CK2 inhibitor, as a potential dual inducer of nucleolar stress and

immunogenicity (Fig. 6a). Next, we determined whether nucleolar stress inducers drive heterochromatin disengagement and augment immunogenicity. Classical nucleolar stress inducers (NSI) comprise low-dose actinomycin D (LAD) and CX-5461, which both inhibit RNA polymerase I³⁸. Of note, CX-4945 disrupted the colocalization of NPM1 with the H3K9me3-marked heterochromatin complex, leading to the redistribution of H3K9me3 towards the nucleoplasm (Fig. 6b, c; Supplementary information, Fig. S7a). Transcriptomic analyses of CX-4945-treated cells revealed a tangible upregulation of numerous RE (Supplementary information, Fig. S7b, c), along with elevated expression of genes involved in viral response pathways, type I interferon signalling, and antigen presentation (Supplementary information, Fig. S7d). Furthermore, both CX-4945 treatment and genetic silencing of CK2 complex components induced the expression of ISGs, MHC-I/HLAAP genes, and RE, ultimately enhancing MHC-I/HLA surface expression in both human and mouse cancer cells (Supplementary information, Fig. S7e-i). Induction of nucleolar stress by genetic or pharmacological manipulations mimicked the effects of CX-4945 (Supplementary information, Fig. S7e, f, j-o). We conclude that nucleolar stress inducers, such as CX-4945, elicit heterochromatin disengagement, which subsequently derepresses RE transcription and amplifies tumour immunogenicity.

To investigate the impact of CX-4945 on TIME, we conducted scRNA-seq analysis of CD45⁺ immune cells isolated from YUMMER1.7 tumours in mice treated with or without CX-4945 (Fig. 6d-g). CX-4945 treatment led to a significant intratumoral expansion of myeloid cell populations, especially CD86⁺ and MHC-II⁺ Mφ. Additionally, CX-4945 treatment enhanced the relative abundance of NK, CD4⁺ and CD8⁺ T cells compared to control conditions (Fig. 6d, e). CX-4945 treatment was associated with the enrichment of gene sets related to inflammatory responses and TNFα signalling via NF-κB in Mφ, as well as α/β T cell activation and IL-2 production by T cells (Fig. 6f, g). These findings highlight a tumour-extrinsic effect of CX-4945 leading to substantial remodelling of both the myeloid and lymphocyte compartments suggestive of a "hot" TIME.

To further evaluate the immunogenic potential of CX-4945, we assessed its capacity to enhance immunogenicity in vaccination assays. As compared to unvaccinated controls, mice vaccinated with CX-4945-treated cells exhibited retarded tumour growth, with some achieving complete resistance to tumour formation (Fig. 6h, Supplementary information, Fig. S7p). CX-4945 also sensitized YUMMER1.7 and MCA205 tumors to PD-1 blockade (Fig. 6i, Supplementary information, Fig. S7q), confirming its immunostimulatory action.

Finally, we evaluated whether inhibition of the OTUD4-NPM1 interaction with OIP could be used to enhance the efficacy of anti-PD1 blockade. OIP monotherapy significantly reduced YUMMER1.7 and MCA205 tumour growth, sensitizing them to anti-PD1 therapy (Fig. 6j, Supplementary information, Fig. S7r). Similarly, in severely immunodeficient NCG mice reconstituted with human peripheral blood mononuclear cells (huPBMCs), OIP administration suppressed the growth of patient-derived xenograft (PDX) tumors and augmented the therapeutic efficiency of anti-PD-1 treatment (Fig. 6k). Immunostaining of such PDX confirmed that OIP monotherapy markedly increased HLA expression by cancer cells and promoted CD8⁺ T cell infiltration into the tumour bed (Fig. 6l). These findings collectively indicate that the disruption of OTUD4–NPM1 interaction recapitulates the phenotypes of OTUD4 loss and potentiates antitumor immunotherapy (Fig. 6m).

***OTUD4* amplification in human tumors**

To examine the clinical impact of OTUD4, we analysed data from the cBioPortal and The Cancer Genome Atlas (TCGA). Genomic data from cBioPortal revealed *OTUD4* amplification in specific cancer types, including pancreatic neuroendocrine tumor (panNET), uterine endometrioid carcinoma (UEC) and chromophobe renal cell carcinoma (ChRCC) (Fig. 7a). To investigate the effect of OTUD4 on the TIME in clinical samples, we reanalysed a public scRNA-seq dataset on panNET (Fig. 7b-f). Notably, *OTUD4*-low tumour specimens exhibited significantly higher levels of transcripts indicating the presence of T cells, NK cells, monocytes, DCs and Mφ, along with reduced levels of transcripts from cancer-associated fibroblasts (CAFs) (Fig. 7c,

d). Concurrently, Mφ displayed activated type I IFN signalling (Fig. 7e), and T cells demonstrated increased proliferative capacity but diminished terminal exhaustion (Fig. 7f). Furthermore, high *OTUD4* expression inversely correlated with MHC-I scores (Fig. 7g) and activated CD8⁺ T lymphocytes signatures across multiple cancers (Fig. 7h). Collectively, these findings suggest that *OTUD4* amplification in malignant cells results in immune exclusion. Consistently, elevated *OTUD4* expression was associated with poor overall survival in patients of low-grade gliomas (LGG), adrenocortical carcinoma (ACC), and pancreatic adenocarcinoma (PAAD) (Fig. 7i, j). These findings established *OTUD4* amplification as a high-risk factor implicated in immune evasion and adverse clinical outcomes across multiple malignancies.

Next, we conducted a two-dimensional transcriptome screening to delineate *OTUD4*-dependent gene expression networks in clinical samples ((Fig. 7k-m). In the first dimension, we identified *OTUD4*-correlated genes in low-grade glioma (LGG), revealing 2,906 genes whose mRNA expression levels were associated with *OTUD4* expression (Fig. 7k). Notably, these genes were enriched in the nuclear, chromosomal and nucleolar regions, with GO analyses indicating their involvement in processes such as ribosome biogenesis, rRNA metabolism and ribonucleoprotein complex assembly (Fig. 7l). In the second dimension, we monitored nucleolar-localizing proteins identified by Andersen et al³⁹, identifying 182 nucleolar proteins correlating with *OTUD4* expression in LGG patients, which we defined as the *OTUD4*-nucleolus score. A high *OTUD4*-nucleolus score was associated with poor survival in patients with LGG (Fig. 7m). Because *OTUD4* regulates transcription indirectly through heterochromatin remodelling, we sought to capture its functional deficiency not by *OTUD4* expression itself but through a derived *OTUD4*-KO gene expression signature from RNA-seq data. Specifically, the most differentially expressed genes following *OTUD4* loss were selected as signature genes, and this *OTUD4*-KO signature score was then evaluated in clinical cohorts. Notably, patients with urothelial carcinoma who responded to anti-PD-L1 therapy exhibited markedly elevated *OTUD4*-KO signature scores (Fig. 7n). These findings suggest that nucleolar *OTUD4* indeed subverts the immunotherapy responses.

749

750 **DISCUSSION**

751 Through CRISPR-based functional screens, we identified the nucleolar deubiquitinase
752 OTUD4 as a topological guardian of heterochromatin architecture. Our data indicate
753 that OTUD4 deficiency compels nucleolus-anchoring, H3K9me3-marked
754 heterochromatin towards the nucleoplasm. This structural relocation derepresses
755 retroelements, thus activating dsDNA sensor signaling and eventually eliciting immune
756 responses. Mechanistically, OTUD4 interacts with NPM1 through a C-terminal R-rich
757 SLiM, deubiquitinates K63-linked ubiquitin at the NPM1 K27 site, and stabilizes its
758 oligomerization. OTUD4 loss disrupts NPM1 oligomerization, weakening the tethering
759 of H3K9me3-marked heterochromatin to NPM1, ultimately releasing perinucleolar
760 H3K9me3. The K63-specific deubiquitinase activity of OTUD4 is regulated by CK2-
761 mediated phosphorylation, with pharmacological inhibition of CK2 using CX-4945
762 phenocopying OTUD4 loss. From a translational perspective, we identify multiple
763 pharmacological inducers of perinucleolar heterochromatin disengagement—including
764 OTUD4-targeting peptides, CK2 inhibitor CX-4945, NPM1 oligomerization inhibitor
765 NSC348884, and RNA polymerase I/II inhibitors—trigger perinucleolar
766 heterochromatin disengagement. These interventions restore tumor immunogenicity
767 and significantly sensitize malignancies to immunotherapy. Collectively, our study
768 unveils the spatiotemporal sequestration of heterochromatin as a central mechanism
769 restraining tumor immunogenicity, and highlight the reconfiguration of perinucleolar
770 heterochromatin topology as a potential therapeutic strategy for stimulating cancer
771 immunosurveillance, with broader implications for aging, viral infection, and
772 autoimmunity (Supplementary information, Fig. S8).

773

774 The spatial architecture of the genome represents a fundamental regulatory stratum
775 that governs cell identity and stress adaptation. Our study positions the nucleolus as
776 an architectural cornerstone of the genome and a pivotal integrator of cellular stress.
777 Beyond its canonical roles in ribosome biogenesis, our findings define the nucleolus
778 as a central spatial rheostat that tempers tumor-intrinsic immunogenicity by
779 orchestrating the sequestration of heterochromatin to serve as a dynamic

"immunological anchor." While the nuclear lamina has long been recognized as a primary bastion of gene repression⁶, our data unveil perinucleolar heterochromatin is not a passive repository of silenced DNA but a highly structured, signal-responsive compartment whose spatial integrity is paramount for preventing aberrant innate immune activation. Central to this 3D genomic organization are nucleolus-associated domains (NADs), which occupy a unique niche within the heterochromatic landscape⁷. For example, pericentromeric repeats disengage from the nucleolus in aging cells⁴⁰, while oncogenic translocations such as CCND1 and MYC relocalize to perinucleolar regions where nucleolin promotes their activation⁴¹. We demonstrate that the assembly of these domains is not an invariant default state but an exquisitely regulated topological program. We identify the CK2–OTUD4–NPM1 axis as the mechanochemical bridge coupling nucleolar integrity to heterochromatin anchoring. While prevailing models emphasize the role of phase-separated condensates in nuclear compartmentalization⁴², our data introduce a precise biochemical trigger: CK2-mediated phosphorylation of OTUD4 acts as a molecular switch, shifting its deubiquitinase specificity toward K63-linked chains on NPM1, thereby stabilizing the NPM1 pentameric scaffold required to physical anchor H3K9me3-marked heterochromatin. Remarkably, disrupting this cascade triggers a selective "disengagement" of perinucleolar heterochromatin without an immediate erasure of repressive histone marks. This decoupling suggests that the 3D genome is hypersensitive to signal transduction, allowing the nucleolus to rapidly recalibrate the repressive landscape in response to oncogenic cues. Critically, this spatial reorganization—rather than global epigenetic attrition—is the primary catalyst for tumor immunogenicity. The detachment of NADs precipitates a localized topological collapse, resulting in the catastrophic derepression of endogenous retroelements. This derepression induces a state of "viral mimicry," characterized by the cytoplasmic accumulation of dsDNA, engagement of the cGAS–STING pathway, and the restoration of MHC-I-mediated antigen presentation. These observations align with an emerging paradigm where the breakdown of nuclear architecture converts genomic stress into a potent innate immune signal. By sequestering these genomic "pro-adjuvants," the nucleolus acts as a gatekeeper of tumor immunogenicity. Its structural

disruption effectively transforms "cold" immunosuppressive microenvironments into "hot" immunogenic ones by unmasking an endogenous reservoir of molecular adjuvants.

As a member of the OTU DUB family, OTUD4 has been implicated in a diverse array of physiological and pathological processes, including zebrafish embryonic development⁴³, cancer progression^{44–47}, ataxia, dementia and hypogonadotropism in human patients⁴⁸. Previous studies have established its roles in regulating antiviral innate immunity⁴⁹, NF- κ B signaling¹⁵, DNA damage repair following DNA alkylation⁴⁴, functioning as an RNA-binding protein within RNA granules²¹ and stabilizing the H3K9me3 methyltransferase SETDB1⁴⁷. Emerging evidence also suggests a potential involvement of OTUD4 in translation-related processes, as its deficiency reduces global translation rates by impairing polysome assembly and increasing eIF2 α phosphorylation, effectively halting translation²¹. In addition to these functions, our findings reveal that OTUD4 deficiency disrupts nucleolar functionality, impairing pre-rRNA processing and ribosome assembly. Mechanistically, OTUD4 deubiquitinates K63-linked NPM1 to maintain nucleolar integrity and dynamics. Furthermore, OTUD4 ablation instigated the derepression of endogenous retroviruses (ERV). In contrast, within the nuclear compartment, OTUD4 interacts with and stabilizes the replication and transcription activator (K-RTA) to awaken Kaposi's sarcoma-associated herpesvirus (KSHV)⁴⁵. In the context of immune regulation, OTUD4 has been shown to deubiquitinate and stabilize the immune checkpoint protein CD73, thereby suppressing antitumor immune responses and facilitating immune evasion⁵⁰. In parallel, OTUD4 stabilizes lactate dehydrogenase A (LDHA), enhancing lactate production in the presence of glutamine. Conversely, OTUD4 deletion, glutamine deprivation, or inhibition of glutamine transport triggers IFN β responses, augments CD8⁺ T cell infiltration, and impairs tumor growth⁵¹. Consistently, our findings demonstrate that OTUD4 depletion amplifies tumor immunogenicity, thereby inflaming TIME and effectively suppressing tumor progression. Collectively, these results highlight the cancer-promoting functions of OTUD4 as surveillance of nucleolar homeostasis.

842

843 From a translational perspective, these insights delineate a conceptual framework for
844 "spatial epigenetic therapy." Most patients with solid tumors derive limited long-term
845 benefit from ICB therapy. Resistance mechanisms include diminished CD8+ T cell
846 infiltration, impaired interferon signaling or antigen presentation, and the loss of tumor
847 antigens. These nucleolar stress inducers to disrupt the heterochromatin-nucleolus
848 interface, augmenting immunogenicity and synergizing with PD-1 blockade. Targeting
849 this subnuclear architecture offers a refined approach to overcoming immune evasion
850 in "excluded" or "cold" tumors. CX-4945, an orally bioavailable small-molecule inhibitor
851 of protein kinase CK2, has been investigated in preclinical and clinical trials for its
852 potential to treat a range of cancers, including solid tumors and hematological
853 malignancies, with particular promise in cholangiocarcinoma (CCA). CCA is a rare and
854 aggressive bile duct malignancy and frequently diagnosed at an advanced stage, and
855 current treatment options (such as surgery, chemotherapy, and radiation) have limited
856 efficacy, particularly in metastatic disease⁵². Approved as an orphan drug by the FDA
857 in 2017 for the treatment of CCA, CX-4945 has demonstrated favorable tolerability and
858 substantial therapeutic efficacy in early-phase clinical trials, and then sensitized
859 patients with CCA to chemotherapy agents like gemcitabine^{53,54}. From an immune-
860 regulatory perspective, CK2 phosphorylated PD-L1 at Thr285 and Thr290 to stabilize
861 PD-L1 in cancer and dendritic cells, thereby inhibiting T cell function⁵⁵. Inhibition of
862 CK2 significantly reduces the accumulation of polymorphonuclear myeloid-derived
863 suppressor cells and tumor-associated macrophages, thereby enhancing the
864 antitumor activity of ICB⁵⁶. Parallely, our findings indicated that CX-4945 serves as a
865 nucleolar stress inducer, enhancing tumor immunogenicity and subsequently
866 synergizing with the antitumor effects of ICB. Thus, the combination of CX-4945 with
867 ICB warrants further investigation in clinical trials across multiple cancer types,
868 particularly for patients with panNET and CCA.

869

870 In summary, the CK2–OTUD4–NPM1 axis dictates a critical architectural checkpoint
871 that masks the malignant genome from innate surveillance. Our work refines the
872 understanding of how nuclear compartmentalization shapes pathophysiological

outcomes and introduces the possibility of reprogramming immune resistance by modulating the spatial epigenetic landscape. Beyond oncology, this mechanism of spatial sequestration likely represents a conserved evolutionary strategy for maintaining genomic and immunological homeostasis.

Limitations of the study

Our study acknowledges several limitations that warrant further investigation. First, although our findings may inform therapeutic strategies for tumors with MHC-I suppression resulting from gene regulatory mechanisms, they may not be applicable to tumors harboring genetic deletions of MHC-I components or loss of HLA gene heterozygosity. Second, while we utilized a diverse pharmacological toolkit—comprising the CK2 inhibitor CX-4945, the NPM1 inhibitor NSC348884, and our engineered OTUD4-interfering peptide (OIP)—the inherent risk of off-target effects associated with these small molecules and peptides cannot be entirely dismissed. Future studies employing more selective chemical biology tools, such as proteolysis-targeting chimeras (PROTACs) or higher-affinity bio-orthogonal probes, will be necessary to definitively confirm the therapeutic window of this axis. Third, OTUD4 is a multifaceted deubiquitinase with established roles in RNA granule dynamics, DNA damage repair, and translational control. Although our findings highlight the NPM1-heterochromatin axis as a primary driver of immunogenicity, we cannot rule out the possibility that other pleiotropic functions of OTUD4 contribute to the overall immune-sensitive phenotype observed in our models. Fourth, beyond RE-derived immunogenicity, the enrichment of antigen-presenting cells such as DCs and macrophages in OTUD4-deficient tumors suggests that other systemic pathways may be at play. Identifying specific RE-derived antigens induced by OTUD4 loss will be crucial for understanding the full scope of immune recognition in this context. Finally, a significant technical constraint is the current lack of a validated antibody specific to the phospho-activated form of OTUD4 (pS202/pS204). Consequently, our clinical correlations were restricted to mRNA expression levels and an OTUD4-KO gene signature rather than the direct quantification of activated OTUD4 protein in patient specimens. Developing such a high-fidelity tool is essential for identifying patient

cohorts most likely to benefit from spatial epigenetic therapies.

METHODS AND METHODS

Cell lines and general materials

MCA205 (fibrosarcoma), B16F10-OVA (mouse melanoma) and B3Z lines were generously provided by Guido Kroemer (Centre de Recherche des Cordeliers), while CT2A (glioma) cells were a kind gift from Yu Cheng (Tongji University). K562 (human chronic myelogenous leukemia) cells were a kind gift from Zhi Li (The first affiliated hospital of USTC). YUMMER1.7, YUMM1.7 (mouse melanoma), B16F10, LLC (mouse Lewis lung carcinoma), MC38 (colon cancer), 4T1 (murine mammary carcinoma), A549 (human lung adenocarcinoma), HeLa (human cervical carcinoma), U2OS (human osteosarcoma) and RCC4 (human clear cell renal cell carcinoma) were obtained from the American Tissue Culture Collection (ATCC). All cell lines were tested for mycoplasma contamination and cultured in RPMI (Thermo Fisher Scientific) or DMEM (Thermo Fisher Scientific) media, supplemented with 10% fetal bovine serum (FBS) and antibiotics. The agents utilized in this study included α -amanitin (AMN, MedChemExpress (MCE #HY-19610), actinomycin D (MCE #HY-17559), NSC348884 (MCE #HY-13915), MG132 (MCE #HY-13259), zidovudine (MCE #HY-17413), and nevirapine (MCE #HY-10570).

Animal experiments

Male C57BL/6J and female BALB/c wild-type mice, along with athymic male nude mice and OT-I mice, were obtained from Charles River Laboratories. *Ifnar1*^{-/-} C57BL/6J mice were sourced from GemPharmatech. NCG mice (NOD/ShiLtJGpt-*Prkd^{cem26Cd52}//2rg^{em26Cd22}*) with human peripheral blood mononuclear cells (huPBMC) were obtained from Shanghai Model Organisms Center. All animal experiments were conducted with approval from the Ethics Committee of Shanghai Tenth People's Hospital (project number SHDSYY-2022-E0002) and were performed in accordance with institutional and governmental ethical guidelines. Mice were housed in a temperature-controlled, specific pathogen-free environment under a 12-hour light/dark cycle with ad libitum access to food and water. Tumor inoculation experiments were

performed using age-matched mice aged 7–8 weeks. Group sizes were determined based on prior experimental experience.

Mice were randomized into experimental groups, matched for age, sex, and genetic background; however, blinding was not performed. To establish tumor models, YUMMER1.7 (1×10^6), YUMM1.7 (5×10^5), B16F10 (5×10^5), MCA205 (5×10^5), MC38 (1×10^6), CT2A (1×10^6), LLC (5×10^5), and 4T1 (5×10^5) cells were injected subcutaneously (s.c.) or into the mammary fat pad of C57BL/6J or BALB/c host mice. Once tumor sizes reached $\sim 20 \text{ mm}^2$, mice were treated via oral gavage with CX-4945 (50 mg/kg), dissolved in a mixture of 0.1% Tween 80, 0.5% sodium carboxymethyl cellulose (NaCMC), and 20% hydroxypropyl-beta-cyclodextrin, administered twice daily. Control groups received an equivalent volume of vehicle solution. CD8⁺ T-cell depletion was achieved by intraperitoneal injection of 200 μg of anti-mouse CD8 α antibody (clone 2.43, Bio X Cell) or control rat IgG (IRTSDIGGGFLY100MG, Innovative Research). Macrophage depletion was performed using clodronate liposomes (FormuMax Scientific), with control liposomes as a reference, delivered via *i.p.* injection of 150 μL of a 7 mg/mL solution. For immunotherapy experiments, mice received 200 μg of anti-PD-1 antibodies (clone RMP1-14, Bio X Cell) or control rat IgG, also administered by *i.p.* injection.

For the immune memory experiments, control or *Otud4*^{-/-} YUMMER1.7 cells (1×10^5) were subcutaneously injected into the left flank of C57BL/6J host mice once per week, for a total of two injections. Two weeks later, control or *Otud4*^{-/-} YUMM1.7 cells (5×10^5) or MCA205 cells (5×10^5) were injected into the contralateral flank, and tumor growth was monitored over the subsequent weeks. For anticancer vaccination studies, the standard protocol described previously was followed⁵⁷. Briefly, YUMMER1.7 or MCA205 cells were treated for 24 hours to achieve 50–70% mortality, after which 1×10^6 cells were resuspended in 200 μL PBS and injected s.c. into the left flank of immunocompetent C57BL/6 mice. Two weeks later, live YUMMER1.7 (1×10^6) or MCA205 (5×10^5) cells were injected into the contralateral flank, and tumor growth was monitored over the following weeks.

966

967 For patient-derived xenograft, the extent of reconstitution huPBMC-NCG mice was
968 determined by flow cytometry after three weeks (Supplementary information, Fig. S1).
969 Tumor tissues were cut into were cut into 3 x 3 x 3 mm fragments under sterile
970 conditions and mixed 1:1 with Matrigel (Corning #356255) to facilitate engraftment.
971 Mice were anesthetized with isoflurane, and tumor fragments were implanted
972 subcutaneously into the flank region using 13G Trocar. The site was closed with
973 surgical clips or sutures, and mice were monitored until recovery. Mouse well-being
974 and tumor growth were assessed every 2–3 days using digital calipers. Tumor area
975 was calculated as: $\text{Area} = \text{Width} \times \text{Length} \times 3.14/4$. Animals were euthanized upon
976 reaching ethical tumor size endpoints or when signs of significant distress were
977 observed, in accordance with EU Directive 63/2010 and the recommendations of the
978 Ethics Committee.

979

980 **Human clinical samples**

981 Two primary breast carcinomas (BC) used for patient-derived xenograft (PDX) mouse
982 models separately were collected from Renmin Hospital of Wuhan University and
983 Hubei Cancer Hospital, with the informed consent of patients and with the approval of
984 the Institutional Review Committees of Renmin Hospital of Wuhan University (project
985 number WDRY2021-Y065) and Hubei Cancer Hospital (project number 2025-A024).
986 Two primary BC used for human breast cancer organoids were collected from Fudan
987 University Shanghai Cancer Center, with the informed consent of patients and with the
988 approval of the Institutional Review Committees of Fudan University Shanghai Cancer
989 Center. The research is not intended to provide direct medical or financial benefit to
990 anyone. Characteristics of two patients used for PDX models were listed in
991 Supplementary information, Table S1.

992

993 **CRISPR Screens**

994 To identify regulators of perinucleolar heterochromatin, we performed CRISPR-based
995 screens for modulators of H3K9me3 abundance. Because cell-cycle arrest can perturb
996 nucleolar–heterochromatin interactions, all screens were conducted in asynchronously

growing, untreated cells. A total of 5×10^7 B16F10–OVA cells were transduced with a custom nucleolomics-focused CRISPR library targeting 659 nucleolar-associated genes (3,954 sgRNAs), together with non-targeting controls, at a low multiplicity of infection (MOI = 0.3) to ensure single-guide integration. This library focused on nucleolar-localizing proteins identified in previous studies^{39,58}. Following transduction, the cells were cultured for 2 days in DMEM-supplemented media, selected with 4~8 $\mu\text{g/mL}$ puromycin for 4 days, and further cultured for an additional 2 days to deplete residual protein from targeted genes. Cells harvested for flow cytometry were washed in PBS and resuspended with PBS at a concentration of 1.5×10^7 cells/ml in 15 ml tubes. Cell suspension was dropwise added into the ethanol (maintaining a PBS to ethanol ratio of 3:7) and agitated for 10 min at 4°C. Cells were centrifuged at 500 g, 4°C for 5 min and washed with 20 ml PBS containing 1% bovine serum albumin (BSA). The cells were then resuspended in 0.5% Triton X100 in PBS for 15 min at 4 °C. After centrifugation, the supernatant was removed and the cells were blocked with 3% BSA in PBS for 30 min at room temperature (RT). The H3K9me3 antibody at a dilution of 1:250 was incubated for 2 hours at RT, followed by incubation with a secondary antibody (AF488) at a dilution of 1:500 for 30 minutes at RT in the dark. Subsequent to primary and secondary antibody staining, the cells were washed with 0.2% Triton X-100 in PBS (with 1% BSA). The samples were then resuspended in PBS (with 1% BSA) and used for FACS. The top 10% and tail 10% H3K9me3 staining cells were collected and subjected to DNA extraction. Genomic DNA from sorted populations was subjected to sgRNA amplification and deep sequencing at ~300× library coverage.

Data analysis was conducted using MAGeCK software, which quantified and assessed sgRNA and gene enrichment. Sequence reads were trimmed to remove constant regions of the sgRNA sequences and mapped to the mouse CRISPR library using MAGeCK's "count" module to calculate read counts. The "test" module was then employed to identify regulators by determining sgRNAs with significant enrichment in the sorted populations compared to the unsorted controls. For genes with negative fold changes—deemed irrelevant in flow cytometry (FACS)-based CRISPR screens—a log fold change of zero was assigned. Mean log fold changes across screens were

computed and visualized using the MAGeCKFlute R package. To identify positive regulators, gRNA distribution in the low-expression population was compared with the unsorted control or high-expression population. Conversely, to identify negative regulators, gRNA distribution in the high-expression population was compared with the unsorted control or low-expression population. These analyses enabled the identification of candidate genes involved in the expression regulation (Supplementary information, Table S2-3).

Plasmids, cell transfection and viral transductions

Stable knockout (KO) of *OTUD4*, as well as *IRF1*, *TET2*, *B2M*, *STING*, *MAVS*, *TRIM28*, and *SETDB1*, in various cell lines was achieved using lentiviruses produced in HEK293T cells. These lentiviruses were generated with lentiCRISPR-v2 vectors containing specific single-guide RNAs (sgRNAs) and selected via puromycin or blasticidin resistance. Stable knockdown experiments employed lentiviral short hairpin RNAs (shRNAs) targeting *OTUD4*, *NPM1*, *POLR1A*, *POLR2A*, *NPM1*, *CSNK2A1*, and *CSNK2B*. The YUMMER1.7-OVA cell line was generated through lentiviral transduction with pCDH-EF1a-OVA. Full-length human *OTUD4* cDNA was used as a template for generating mutants, as previously described⁴⁹. HA-tagged NPM1 plasmids, generously provided by Xinyi Lu (Nankai University), were PCR-amplified and cloned into expression vectors using seamless cloning. His-tagged ubiquitin constructs—wild-type (His-Ub wt), K48O/K48R mutants, and K63O/K63R mutants—were generated in our previous study⁵⁹. The canonical NoLS from p65 (sequence RKKRKKK) was incorporated into OTUD4 truncations through PCR. Chimeric constructs, mutations, and truncations in OTUD4 or NPM1 were introduced by PCR, cloned into pCDH-Flag or pCDH-HA vectors. For recombinant protein production, *OTUD4* fragments were cloned into the pCDH-Flag vector. All PCR-derived constructs, including deletions and point mutations were validated by Sanger sequencing.

To activate endogenous L1MEC elements, we create a cell line permanently expressing the dCas9-VPR. Non-targeting control sgRNA (sgNTC) or sgRNA targeting the L1MEC (sgL1MEC) was cloned into the lentiCRISPR-v2 vector. For endogenous

repression of L1MEC, constructs encoding dCas9-KRAB and sgL1MEC (CRISPRi-sgL1MEC), as well as the dCas9-KRAB-expressing empty vector control (CRISPRi-control). We used Benchling (<https://benchling.com/>) to identify sgRNA target sites. To assess the specificity of the sgRNAs for endogenous L1MEC elements, we first identified genomic loci annotated as L1MEC by RepeatMasker using the mouse genome assembly (mm10). Through sequence alignment of the sgRNA with L1MEC elements in the mouse genome, we confirmed that the designed sgRNAs selectively target three potentially full-length L1MEC elements with high specificity for use in both CRISPRa and CRISPRi systems. Detailed sequences of sgRNAs and shRNAs are provided in Supplementary information, Table S4.

Protein purification

Recombinant OTUD4 Flag-tagged proteins were purified from 293F cells using an ÄKTA Pure fast protein liquid chromatography (FPLC) system (GE Healthcare). The cells were resuspended in lysis buffer (25mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM TCEP, and protease inhibitors) and lysed via sonication. The resulting lysate was clarified by centrifugation, filtered, and loaded onto a FlagTrap HP column (GE Healthcare). After extensive washing with lysis buffer, the bound proteins were eluted with lysis buffer supplemented with 3 mg/ml flag peptide. The eluates were subsequently dialyzed into PBS buffer.

Interfering peptides

The OIP peptide was modified with the TAT sequence (*YGRKKRRQRRRFRGDRRRSG*; molecular weight: 2,648 g/mol; TAT sequence indicated in italics). As a control, a peptide with the identical amino acid composition but randomized sequence—also conjugated with TAT—was used (*YGRKKRRQRRRDRSRGRFRG*; TAT sequence in italics). Both peptides were synthesized by QYAOBIO (ChinaPeptides) at >95% purity and stored at -20 °C in 2 mg powder aliquots to prevent degradation from freeze-thaw cycles. For *in vitro* applications, peptides were reconstituted in PBS to a final concentration of 25 mM. For *in vivo* studies, peptides were dissolved in PBS at 4 mg/ml, maintained on ice until

injection, and equilibrated to room temperature immediately prior to administration.

Organoid culture

Tumor specimens were obtained either by 18G core needle biopsy or surgical resection and processed for organoid culture within 24 hours of collection. Biopsy-derived tissues were mechanically dissociated into small fragments using sterile needles and embedded in Geltrex (LDEV-free, reduced growth factor basement membrane extract; GIBCO). Resected tumor tissues, in contrast, were finely minced and enzymatically digested in a solution containing 1.5 mg/mL collagenase II, 10 mg/mL hyaluronidase type IV, and 10 mM Y-27632 (all from Sigma-Aldrich). The resulting cell suspensions were embedded in Geltrex, allowed to solidify for 20 minutes at 37 °C, and subsequently overlaid with human organoid medium. To reduce microbial contamination during the early stages of culture, 1× Primocin (InvivoGen) was added for the first two weeks. Organoids were passaged approximately once per week by enzymatic dissociation with TrypLE Express (GIBCO) at 37 °C for 5–10 minutes, followed by re-embedding in fresh Geltrex to enable long-term propagation.

Population-level pre-rRNA pulse-chase

Nascent rRNA synthesis was measured using the Click-iT™ Nascent RNA Capture Kit (Invitrogen, catalog no. C10365). Cells were seeded in six-well plates at a density of 5×10^5 cells per well and cultured to 40–50% confluence. After 24 hours, cells were pulse-labeled with 0.15 mM ethynyl uridine (EU) for 1 hour, followed by a 2.5-hour chase in EU-free medium. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, catalog no. 74104). For click chemistry labeling, 1 µg of total RNA was incubated with 25 µl Click-iT EU buffer, 4 µl CuSO₄, 1.25 µl biotin azide, and 1.25 µl Click-iT reaction buffer additive 1 at room temperature for 3 minutes. Subsequently, 1.25 µl Click-iT reaction buffer additive 2 was added, and the mixture was incubated for an additional 30 minutes. The reaction was terminated by ethanol precipitation in the presence of 1 µl UltraPure™ Glycogen (Roche, catalog no. 10901393001), 50 µl of 7.5 M ammonium acetate, and 700 µl of chilled 100% ethanol. Samples were stored at –80 °C overnight. RNA was pelleted by centrifugation at 13,000g for 20 minutes at 4 °C

and washed twice with 700 µl of 75% ethanol. Next, 1 µg of RNA was mixed with 31 µl Click-iT RNA binding buffer and 2 µl RNaseOUT™ (Thermo Fisher), then heated at 68–70 °C for 5 minutes. The denatured RNA was incubated with 12 µl of pre-washed streptavidin magnetic beads at room temperature for 30 minutes. Beads were then washed five times with Click-iT wash buffer 1 and five times with wash buffer 2. To elute captured RNA, beads were resuspended in 12 µl of wash buffer 2 and incubated at 68–70 °C for 5 minutes. Purified RNA was immediately subjected to qPCR. Pre-rRNA processing efficiency was assessed by comparing levels of unprocessed pre-rRNA containing the 5' external transcribed spacer (ETS) with total mature rRNA levels.

Flow cytometry (FACS) analysis

Cells were washed with FACS buffer (PBS + 2% FBS) and incubated at room temperature for 15 minutes with either Human TruStain FcX (BioLegend #422302, 1:100 dilution) or Mouse TruStain FcX (BioLegend #101320, 1:100 dilution) to block Fc receptor binding. Subsequently, the cells were stained with fluorescently labeled antibodies, including APC-HLA-A/B/C (BioLegend #311410, 1:100 dilution) or PE-H-2 (BioLegend #125506, 1:100 dilution). YUMMER1.7-OVA cells were stained with APC-H-2Kb bound to SIINFEKL (BioLegend #141605, 1:100 dilution) for 30 minutes at 4°C. Data acquisition was performed using a BD LSRFortessa Cell Analyzer or BD LSR II Flow Cytometer and analyzed with FlowJo software.

Between days 10 and 14 post-tumor injection, tumors were excised, minced, and enzymatically digested with 1 mg/mL collagenase/dispase (Sigma-Aldrich, 10269638001) and 1× DNase I (Qiagen, 79254) at 37°C for 30 minutes. The resulting tumor cell suspensions were filtered through a 70 µm mesh to obtain single cells, which were then stained for flow cytometric analysis. Bulk tumor cells were homogenized in PBS at a concentration of 250 mg/mL based on the initial tumor weight. To analyze tumor-infiltrating lymphocytes (TILs), approximately 50 mg of the tumor suspension was blocked with anti-mouse CD16/CD32 (BioLegend #101320) to prevent nonspecific Fc receptor binding. Myeloid cell markers were analyzed using the following antibodies: PerCP/Cyanine5.5-anti-CD45 (BioLegend #103132, 1:200), APC-anti-CD11b

(BioLegend #101212, 1:100), Brilliant Violet 510-anti-CD11c (BioLegend #117353, 1:100), PE-anti-F4/80 (BioLegend #123110, 1:100), FITC-anti-CD86 (BioLegend #105005, 1:100), Brilliant Violet 605-anti-Ly6c (BioLegend # 128035, 1:100), Brilliant Violet 421-MHC-II (BioLegend #107632, 1:100), APC-Cy7-CD103 (BioLegend #121414, 1:50), and Brilliant Violet 711-anti-Ly6g (BioLegend #127643, 1:50). T-cell surface markers were stained using the following fluorochrome-conjugated antibodies: PerCP/Cyanine5.5-anti-CD45 (BioLegend #103132, 1:200), Brilliant Violet 510-anti-CD3 (BioLegend #100233, 1:100), APC-anti-CD8 (BioLegend #100712, 1:100), APC-Cy7-anti-CD25 (BioLegend #101918, 1:100) and FITC-anti-CD4 (BioLegend #100406, 1:200). Staining was performed for 30 minutes at 4°C in the dark. For intracellular staining, the Cyto-Fast Fix/Perm Buffer Set (BioLegend #426803) was used according to the manufacturer's protocol. Samples were then stained with intracellular antibodies, including Brilliant Violet 711-anti-IFN γ (BioLegend #505836, 1:100), Brilliant Violet 421-anti-GZMB (BioLegend #396414, 1:100), Alexa Fluor 700-anti-FOXP3 (BioLegend #126422, 1:200), and PE-anti-Ki67 (BioLegend #151210, 1:100). Data were collected using a BD LSRFortessa Cell Analyzer or BD LSR II Flow Cytometer and analyzed using FlowJo software. Absolute leukocyte and tumor cell counts were normalized based on the tumor weight at harvest, the total volume of the dissociated tumor cell suspension (standardized to 250 mg/mL in PBS), the proportion of the total suspension, and the fraction used for cytometric analysis. These gating strategies were shown in Supplementary Figure 1.

T cell activity assay by B3Z T cell hybridoma co-culture

YUMMER1.7-Cas9-OVA control and *Otud4*-knockout cells were co-cultured with B3Z T cell hybridomas as previously described⁶⁰. In brief, 1×10^5 cells were fixed in 1% paraformaldehyde at 37°C for 10 minutes, followed by quenching with 200 mM glycine (prepared in PBS, pH 7.5) at 37°C for 5 minutes. The fixed cells were washed three times with B3Z medium before 5×10^4 B3Z T cell hybridomas were added per well. Co-cultures were incubated for 10 hours at 37°C. Subsequently, the supernatant was collected, and IL-2 secretion was quantified using an ELISA kit following the manufacturer's instructions (Elabscience #E-EL-M0042).

OT-I T-cell Cytotoxicity Assay

In brief, CD8⁺ T cells were isolated from the spleens and lymph nodes of OT-1 mice and stimulated with anti-CD3/CD28 beads (Thermo Fisher #11452-D), then cultured in complete RPMI 1640 medium supplemented with 10% FBS, 20 mmol/L HEPES, 1 mmol/L sodium pyruvate, 0.05 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 50 U/mL streptomycin, penicillin, and 100 ng/mL recombinant mouse IL-2. To assess the susceptibility of cancer cells to T cell-mediated cytotoxicity in the OT-I model, YUMMER1.7-Cas9-OVA control and *Otud4*-knockout cells were plated at equivalent densities in all wells. T cells were then added at varying ratios to the cancer cells. Following 6 hours of co-culture, cells were lysed and luciferase activity was measured using the LDH cytotoxicity assay Kit (Promega, J2381), according to the manufacturer's protocol. Luciferase activity was normalized to that of cells cultured in the absence of T cells.

qPCR

Total RNA extraction, cDNA reverse transcription and qPCR with reverse transcription were performed as previously described. We used GAPDH to normalize the results and analyzed the data with three replicates. A list of primers for qPCR experiments is provided in Supplementary information, Table S5.

Immunoblotting

Protein extracts were prepared by lysing cells in cold RIPA buffer supplemented with protease and phosphatase inhibitors (MCE #HY-K0010 and HY-K0021). The lysates were sonicated in three 10-second intervals and centrifuged at 12,000 × g for 15 minutes to isolate the supernatant containing soluble proteins. The protein samples were then mixed with 4× loading buffer and denatured at 100°C for 10 minutes prior to Western blot analysis. Approximately 30 µg of total protein per sample was resolved by SDS-PAGE and transferred onto PVDF membranes using a Bio-Rad transfer system. Membranes were blocked with 5% non-fat milk or BSA in TBS containing 0.1% Tween-20 (TBST) for 1 hour at room temperature, followed by overnight incubation

with primary antibodies at 4°C. After incubation, membranes were washed three times for 10 minutes each with TBST and then incubated with species-specific HRP-conjugated secondary antibodies (Pierce) for 1 hour at room temperature. Following three additional 10-minute washes in TBST, membranes were developed using an enhanced chemiluminescence (ECL) reagent and visualized with ECL instrument (Tanon-5200).

For native gel electrophoresis, cells were lysed in RIPA buffer without SDS, supplemented with 1 mM PMSF and a protease inhibitor cocktail, followed by brief sonication. Cell lysates were mixed with 4× Protein Native PAGE Loading Buffer (Beyotime #P0016P) without heating and loaded onto native PAGE gels lacking SDS. Subsequent procedures, including electrophoresis, transfer, and antibody staining, were performed as described for standard SDS-PAGE analysis. The primary antibodies used are detailed in Supplementary information, Table S6, and all raw images of immunoblotting were shown in Supplementary Figure 2.

Immunohistochemistry and immunofluorescence

All experimental procedures adhered to institutional guidelines and local regulations. Immunohistochemical staining was performed using either an automated staining system (Leica Bond3) or manual methods. Tissue sections were dehydrated, and antigen retrieval was carried out in 10 mM citrate buffer by microwave heating for 10 minutes. Specimens were then incubated with primary antibodies specific to CD8 (Cell Signaling Technology #3868, 1:2000), CD4 (Biorbyt #orb503127, 1:100), CD86 (Cell Signaling Technology #93498, 1:500), and F4/80 (Cell Signaling Technology #98377, 1:100). Primary antibody binding was detected using a peroxidase-conjugated IgG secondary antibody (Dako, Glostrup #P0448, 1:500). The images were completely scanned and recorded by Panoramic DESK (3DHISTECH, Hungary). Positive cells were quantified in nine randomly selected tumor regions at 200× magnification, with analysis conducted in a double-blinded manner. Quantitative data were processed using ImageJ software.

For immunofluorescence staining, 25,000 cells were seeded onto poly-L-lysine (PLL)-coated coverslips. Cells were fixed with 1% formaldehyde for 1 minute at room temperature, followed by three washes in 1× PBS (5 minutes per wash). Permeabilization was achieved using 0.3% Triton X-100 for 5 minutes at room temperature, followed by three additional PBS washes. Coverslips were blocked with 5% BSA for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies (diluted in 1% BSA) targeting OTUD4, Flag, NPM, FBL, RPA40, H3K9me2, H3K9me3, H3K27me3, H3K27ac, HP1 α , or dsDNA. After PBS washes, cells were incubated with secondary antibodies (diluted in 1% BSA; 1:800 goat anti-rabbit FITC, 1:800 goat anti-mouse Alexa Fluor 594 or 1:800 goat anti-rabbit Cy5) for 1 hour at room temperature. Coverslips were washed again with PBS, stained with DAPI (1 μ g/mL) for 2 minutes, mounted onto slides with DAKO mounting reagent, and air-dried for 30 minutes. For methanol/acetone fixation, cells were fixed with ice-cold methanol for 15 minutes, rinsed with cold acetone, and washed three times in PBS (5 minutes per wash) before blocking with 5% BSA. Cytoplasmic dsDNA was visualized using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) by incubating cells with 1× PicoGreen reagent for 1 hour at 37°C. Microscopic images were acquired using a Nikon C2⁺ confocal microscope equipped with NIS-Elements AR software (Nikon) at magnifications of 100× or 40×. Cellular debris and dead cells were excluded from analysis. Parameters such as nucleolus count, area, diameter, fluorescence intensity, and localization were quantified using the EBImage R package. Image data were processed and pixel intensities normalized to ensure consistent visibility across all images within a given experiment. Antibodies used for immunofluorescence and immunohistochemistry staining are listed in the key resources Supplementary information, Table S6.

Global translation by AHA incorporation

Translation rates were quantified by measuring the incorporation of L-azidohomoalanine (AHA; Thermo Fisher Scientific #C10289), a methionine analog, using Click-iT chemistry in accordance with the manufacturer's protocol. Briefly, 25,000 cells per well were seeded in 24-well clear imaging plates and allowed to adhere

overnight. The following day, cells were treated with cycloheximide (CHX, 50 μ M; MCE #HY-12320) for 1 hour. After several PBS washes, cells were incubated in methionine-free medium for 30 minutes, followed by an additional 1.5-hour incubation in methionine-free medium supplemented with 25 μ M AHA. Cells were then fixed with 3.7% formaldehyde for 1 hour, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked with 2% BSA for 1 hour. Alexa Fluor 488-conjugated azide was applied for 2 hours to label incorporated AHA. Translation levels were assessed by measuring GFP fluorescence intensity via fluorescence microscopy or flow cytometry. The mean fluorescence intensity (MFI) of GFP was calculated under each condition to determine relative translation rates.

Subcellular fractionation extraction

Cells were washed with PBS, trypsinized, and centrifuged at 1,000 r.p.m. for 5 minutes at 4°C. The resulting pellet was resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl_2 , 0.1% Triton X-100, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 , and protease inhibitors: 1 μ g/mL each of aprotinin, leupeptin, and pepstatin) and incubated on ice for 30 minutes. Following incubation, the sample was centrifuged at $1,300 \times g$ for 5 minutes, and the supernatant was collected as the cytosolic fraction. The nuclear pellet was washed once with buffer A. For chromatin fractionation, the nuclear pellet was lysed by rotation in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, supplemented with the same protease and phosphatase inhibitors) at 4°C for 30 minutes. After centrifugation at $12,000 \times g$ for 20 minutes, the nucleoplasmic supernatant was collected. The insoluble chromatin pellet was washed once with buffer B, centrifuged as described above, and resuspended in 1 $\times$ sample loading buffer. To isolate the nucleolar fraction, the nuclear pellet was resuspended in a solution containing 0.25 M sucrose and 10 mM MgCl_2 and layered over 0.35 M sucrose with 0.5 mM MgCl_2 . This preparation was centrifuged at 1,000 r.p.m. for 5 minutes at 4°C, and the pellet was resuspended in 0.25 M sucrose with 10 mM MgCl_2 . The sample was then sonicated at 25% power for six cycles of 10 seconds, with 10-second intervals on ice. The sonicated sample was layered over 0.88 M sucrose with 0.5 mM MgCl_2 and centrifuged at 3,500 r.p.m. for 10 minutes at 4°C to

collect the nucleoplasmic supernatant. The remaining pellet was resuspended in 0.35 M sucrose with 0.5 mM MgCl₂ and centrifuged at 3,500 r.p.m. for 5 minutes at 4°C, yielding the final nucleolar fraction. Western blotting was performed to assess fraction purity, using TUBULIN, FBL, GAPDH, H3, Lamin A/C, and transcription factor CREB as controls. These markers were enriched in the nucleoplasmic fraction but depleted in the nucleolar fraction, confirming the accuracy of the fractionation.

Polysome fractionation

CHX (100 µg/mL) was added to cell cultures for 5 minutes, followed by washing with PBS. Cells were then lysed in a fractionation buffer containing 5 mM Tris (pH 7.5), 2.5 mM MgCl₂, and 1.5 mM KCl, supplemented with cOmplete ULTRA EDTA-free protease inhibitors (Roche), CHX (100 µg/mL), RNase inhibitor (Promega RNasin, 0.2 U/µL), 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate. Nuclei were removed by centrifugation at 16,000 × g for 7 minutes. The optical density (OD) of the cytoplasmic fraction at 260 nm was measured, and the lysate was diluted to an OD of 10–20 (or 40 for mass spectrometry analysis). A 500-µL aliquot of the diluted lysate was layered onto a 5–50% sucrose gradient prepared in 20 mM HEPES (pH 7.6), 100 mM KCl, 5 mM MgCl₂, 10 µM CHX, protease inhibitors (1/10 dilution), and RNase inhibitor (10 U/mL). The gradient was centrifuged at 36,000 rpm for 2 hours at 4°C using a Beckman SW41Ti rotor, and absorbance at 254 nm (A₂₅₄) of the resulting fractions was measured using a UA-6 UV/VIS detector.

Transmission electron microscopy analysis

Cell monolayers were fixed in 2% glutaraldehyde prepared in 0.1 M Sörensen phosphate buffer for 1 hour at 4°C. During fixation, cells were scraped and centrifuged to form pellets. The pellets were rinsed in ice-cold phosphate buffer for 1 hour, post-fixed in 2% aqueous osmium tetroxide, and dehydrated using a graded ethanol series. The samples were embedded in Epon resin and polymerized at 60°C for 48 hours. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate following standard protocols, and examined using an FEI Tecnai Spirit transmission electron microscope operating at 80 kV. Digital images were captured using an SIS MegaView

III charge-coupled device (CCD) camera.

RNA-seq analysis

Total RNA was extracted using the Qiagen RNeasy Plus Mini Kit and submitted to Tianhao Biotech Company (Shanghai, China) for stranded library preparation. RNA-seq libraries were sequenced to a minimum depth of 2.5×10^7 reads per sample. Adapter sequences were removed using Trimmomatic (v.0.39), and the resulting reads were aligned to the mm10 reference genome using the HISAT2 aligner (v.2.2.1). Repeatmasker annotations for the mm10 genome were retrieved from the UCSC Table Browser, and gene annotations were based on Gencode M4. Reads mapping to annotated repeat regions or genes were quantified using featureCounts (v.1.6.2), and differential expression analysis was performed using DESeq2 (v.1.32). Transposable element (TE) families were classified as long terminal repeats (LTRs, subtyped into RE1, REK, and REL), short interspersed nuclear elements (SINEs), or long interspersed nuclear elements (LINEs) using Dfam (release 3.3). The results are detailed in Supplementary information, Table S7.

To assess the clinical relevance of OTUD4, we constructed a *Otud4*-KO gene signature by selecting the 200 most upregulated and 200 most downregulated genes identified from differential expression analysis. DESeq2 Wald statistics were used as weights, which were subsequently normalized to a -1 to 1 scale using the equation $k_i = w_i / \max(w)$, where k_i represents the normalized weight of the i th gene and w_i is its corresponding Wald statistic. For each input gene expression profile, we then calculated a *Otud4*-KO signature score as a weighted sum of the expression levels of the signature genes, defined by the equation $S = \sum_1^n (k_i \times X_i)$, where S denotes the signature score and X_i the expression level of the i th gene. This score was subsequently used to evaluate the association between *Otud4* deficiency and response to immune checkpoint blockade.

ATAC-seq analysis

For ATAC-seq experiments, 50,000 cells from wild-type or *Otud4*^{-/-} YUMMER1.7 cell

lines were submitted to JiaYing Company for stranded library preparation and processed using established tagmentation protocols. Adapter sequences were trimmed using Trim Galore (v.0.6.6), and reads were aligned to the mm10 reference genome with Bowtie2 (v.2.4.2) using the parameters --no-mixed --no-discordant -X 2000 -N 1 -L 25. Duplicate and low-quality reads were removed with SAMtools (v.1.17) using the filtering parameter -F 1804. Tn5 cut site positions were determined by shifting forward-strand reads by +5 bp and reverse-strand reads by -4 bp. To create a consensus peak set, peaks were called for each replicate using MACS3 (v.3.0.0a6) with the parameters --nomodel -s 1 --shift -75 --extsize 150, and the resulting peaks were merged for subsequent analysis. Differential accessibility analysis was conducted using DESeq2 (v.1.32) with read counts normalized by total library sizes. Significant differentially accessible regions (adjusted $P < 0.05$) were used as the foreground dataset for motif enrichment analysis, while all other ATAC-seq regions in the consensus peak set served as the background.

CUT&Tag-seq analysis

CUT&Tag-seq was performed on 50,000 cells from wild-type or *Otud4*^{-/-} YUMMER1.7 cell lines by JiaYing Company following standardized stranded sequencing protocols. Adapter sequences were trimmed with Trim Galore (v.0.6.6), and reads were aligned to the mm10 or dm6 reference genome using Bowtie2 (v.2.4.2) with the parameters --no-mixed --no-discordant -X 2000 -N 1 -L 25. Duplicate and low-quality reads were filtered using SAMtools (v.1.12) with the parameter -F 1804. Peaks were called for each replicate using MACS3 (v.3.0.0a6) with the parameters -f BAMPE --broad --broad-cutoff 0.1 --nomodel, and a consensus peak set was constructed by merging peaks shared between replicates.

Reads overlapping consensus H3K9me3 or H3K27ac peaks, as well as RE-annotated loci, were quantified using featureCounts (v.1.6.2). Log₂-transformed signal ratios were calculated for each region to compare knockout and wild-type samples. For signal normalization, replicates were pooled, and bigwig files were generated using deepTools (v.3.1.3). Normalized signals were visualized as heatmaps created with

either the plotHeatmap function in deepTools or the ComplexHeatmap R package (v.2.4.3). For metagene profiling, bigwig signals across RE regions were averaged using bwtool (v.1.0). Sequence motifs enriched at H3K9me3 or H3K27ac binding sites were identified using HOMER (v.4.10.4) with the findMotifsGenome.pl function and the parameter -size given. Signal correlation was evaluated using two-tailed Pearson correlation tests.

NAD-seq analysis

Formaldehyde cross-linking and nucleolar isolation were performed as previously described⁶¹. Briefly, cells were cultured under standard conditions and fixed by adding formaldehyde directly to the culture medium to a final concentration of 1%, followed by incubation at room temperature for 10 min. Cross-linking was quenched with 1 M glycine, after which cells were washed twice with ice-cold PBS, collected by scraping into PBS, and pelleted by centrifugation at 200 g for 5 min at 4 °C. Cell pellets were resuspended in high-magnesium (HM) buffer (10 mM HEPES–KCl, pH 7.5; 0.88 M sucrose; 12 mM MgCl₂; 1 mM DTT) and subjected to sonication on ice (two to three 10-s pulses at full power) using a Soniprep 150 (MSE) equipped with a fine probe. Efficient nucleolar release was confirmed microscopically, and nucleoli were isolated by centrifugation at 15,000 g for 20 s, then resuspended in low-magnesium (LM) buffer (10 mM HEPES–KCl, pH 7.5; 0.88 M sucrose; 1 mM MgCl₂; 1 mM DTT). Following a second brief sonication (10 s, full power) and centrifugation at 15,000 g for 20 s, nucleolar pellets were resuspended in 20/2 TE buffer (20 mM Tris–Cl, pH 7.5; 2 mM EDTA) for immediate use or preserved in 20/2 TE supplemented with 50% glycerol, snap-frozen in liquid nitrogen, and stored at –80 °C.

Nucleolus-associated domain (NAD) analysis was performed using NADfinder (v 1.26.0), a Bioconductor package implementing a standardized workflow for NAD-seq data processing. The analysis integrated four core functions—*tileCount*, *log2se*, *smoothRatiosByChromosome*, and *callPeaks*—to combine biological replicates and define high-confidence NADs. Briefly, *tileCount* computed read counts across sliding genomic windows (50 kb window size; 1 kb step) for nucleolar and genomic fractions,

and *log2se* calculated $\log_2$ ratios of read counts or counts per million between paired samples. Chromosome-level baseline correction and signal smoothing were performed using *smoothRatiosByChromosome*, followed by peak identification and replicate integration using *callPeaks*. Unless otherwise indicated, default parameters were used, with the exceptions of *lfc* = $\log_2(1.5)$, *countFilter* = 200, and *cutoffAdjPvalue* = 10^{-3} (10^{-2} for chromosome X, owing to reduced signal-to-noise). Overlap analyses between NADs and additional genomic features—including ATAC-seq and CUT&Tag-seq datasets for H3K9me3 and H3K27me3—were conducted using the *GenomicRanges* package. The *intersect* and *setdiff* functions were applied to identify nucleotide-level overlaps and feature-exclusive regions, respectively.

scRNA-seq analysis

CD45⁺ enriched immune cells were isolated from *Otud4*^{-/-} and control YUMMER1.7 tumors after 11 days of in vivo expansion, as well as from YUMMER1.7 tumors treated or untreated with CX-4945 after 14 days of in vivo growth. For the *Otud4*^{-/-} and control tumor experiment, three control unilateral tumors were collected. Due to the smaller size of *Otud4*^{-/-} tumors, 4–5 tumors were pooled per sample, resulting in three *Otud4*^{-/-} samples. In the CX-4945 treatment experiment, three untreated control tumors and three CX-4945-treated unilateral tumors were collected. Tumors were dissociated into single-cell suspensions as described in the “Flow Cytometry Analysis” section. Immune cells were isolated using density gradient centrifugation with Lympholyte reagent (CedarLane Labs) followed by positive selection for CD45⁺ cells using MicroBeads and a magnetic separator (Miltenyi).

Single-cell isolation and 3' or 5' library preparation were performed using the Chromium Controller and the GEXSCOPE Genomics platform according to the manufacturer's instructions. Demultiplexing, read alignment, filtering, and unique molecular identifier (UMI) counting were carried out using the *CellRanger* pipeline (v.3.0, 10x Genomics). Downstream analyses were conducted in R using the Seurat package. Rigorous quality control was applied by filtering cells based on the number of detected genes and the proportion of mitochondrial and ribosomal transcripts. Cells with fewer than

500 UMIs or 200 detected genes were excluded. Potential doublets and multiplets were removed by filtering cells expressing more than 20,000 UMIs or 5,000 genes. Additionally, cells with mitochondrial transcript levels exceeding 10% or ribosomal transcript levels greater than 30% of total expression were excluded to ensure data quality. Batch effects were mitigated, and datasets were harmonized using the Harmony algorithm. Dimensionality reduction, clustering, and differential expression analysis were performed following the Seurat workflow. Principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) were used for dimensionality reduction, focusing on the top 20 principal components to capture the most significant variance. Differentially expressed genes in each cluster were identified using the *FindAllMarkers* algorithm with the Wilcoxon rank-sum test. Cell cluster annotations were assigned based on established gene markers, enabling the identification and classification of distinct cell populations. Cell identities were determined using published marker gene lists (Supplementary Figure 3).

To characterize the cellular architecture of pancreatic neuroendocrine tumors (panNETs), single-cell RNA sequencing data from 15 primary tumor samples were obtained from the Gene Expression Omnibus (GEO) repository (accession number GSE256136). Computational methods were applied similarly to those described above. PanNET samples were stratified into *OTUD4*-high and *OTUD4*-low groups based on the median *OTUD4* expression in pseudobulk profiles.

Whole-genome bisulfite sequencing (WGBS)

Genomic DNA (200 ng) isolated from *Otud4*^{-/-} and control YUMMER1.7 cells was spiked with 0.5% lambda DNA and subjected to bisulfite conversion using the EZ DNA Methylation-Lightning Kit (Zymo Research). The bisulfite-treated DNA was then processed for library preparation with the Accel NGS Methyl-Seq DNA Library Kit, following the manufacturer's protocol. Pooled DNA libraries were sequenced on the Illumina NovaSeq platform using paired-end reads. Raw FASTQ files were mapped to the mm10 genome assembly using bsmap-2.89 software with parameters '-v 6 -n 1 -q 3 -r 1'. Bisulfite conversion efficiency was estimated using the unmethylated lambda

DNA control. The Mcall module from Model-based Analysis of Bisulfite Sequencing Data (MOABS) was employed to calculate the mCG/CG ratios for each CpG site. Only CpGs with coverage ≥ 5 was included in downstream analyses. Differentially methylated regions (DMRs) were called using the Mcomp module with the parameters ‘-minNominalDif=0.2 minDmcsInDmr 3-maxDistConsDmcs 500’. The standard deviation of these differential methylation levels within each 10-kb bin was also calculated. The P-value for age-related DMRs was determined using a two-sided hypergeometric test, followed by Bonferroni correction for multiple comparisons.

Patient dataset analysis

Batch effect-normalized mRNA expression data, *OTUD4* gene-level copy number, and GISTIC2 thresholded copy number calls were downloaded from cBioPortal (www.cbioportal.org). A final dataset of 9,992 patients with complete data was analyzed. Patients with GISTIC-thresholded copy number calls of 1 (low amplification or copy gain) or 2 (high amplification) were classified as having *OTUD4* amplification. Based on the expression of HLA molecules (HLA-A, HLA-B, HLA-C, and B2M), the HLA score was calculated using the GSVA algorithm. We evaluated the association of *OTUD4* expression with HLA score, activated CD8⁺ T cells Infiltration and patient outcome. Furthermore, gene set enrichment analysis (GSEA) was performed on 18 TCGA cohorts with at least 200 available patients, with downsampling to achieve balanced sample sizes across cohorts. Clinical outcomes and processed genomic data, including normalized RNA-seq and *OTUD4* expression from the IMvigor210 trial ICB-treated cohort (urothelial carcinoma), were obtained from relevant publications (GSE145281). Survival statistics were computed using the log-rank test. Candidate *OTUD4*-correlated and nucleolar-localized genes were identified from The Cancer Genome Atlas LGG dataset using GlioVis and the nucleolar proteome dataset, respectively. Overlapping genes from both lists were subjected to Gene Ontology (GO) enrichment analysis using the topGO package (Bioconductor, R package v.2.32.0). Statistical significance was determined using a hypergeometric test.

Cytoplasmic dsDNA isolation and re-transfection

Equal amounts of *Otud4*^{-/-} YUMMER1.7 cells, with or without treatment with RTi (zidovudine and nevirapine, 20 µM) for 24 hours, were harvested and washed with cold PBS. Cytoplasmic fractions were prepared by removing the nuclear fraction through centrifugation, as detailed in the "Subcellular Fractionation Extraction" section. The cytoplasmic fraction (390 µL) was supplemented with 25 µL of 5 M NaCl and treated with 8 µL of RNase A/T1 Mix (Thermo Fisher Scientific #EN0551) for 30 minutes at 37°C. DNA was subsequently extracted using the Gel Extraction Kit (QIAGEN #28704). Quantification of specific RE DNA in the cytosol was performed by qPCR with primers listed in Supplementary information, Table S5, and normalized to GAPDH levels.

For the cytoplasmic DNA re-transfection assay, 1 µg of cytoplasmic DNA was digested with either 1 µL of DNase I (Thermo Fisher Scientific #EN0521) or H₂O in a 10 µL reaction mixture for 30 minutes at 37°C. Following digestion, DNA was transfected into YUMMER1.7 cells using 1.5 µL of Lipofectamine 2000 (Thermo Fisher Scientific). As a positive control, 0.8 µg of HT-DNA was transfected. After 48 hours, RNA was isolated and analyzed by qPCR using the specified primers.

Co-immunoprecipitation (IP)

Semi-exogenous and endogenous co-immunoprecipitation (Co-IP) assays were conducted using cell lysates prepared by sonication in IP buffer containing 20 mM HEPES (pH 7.8), 400 mM KCl, 5% glycerol, 5 mM EDTA, 0.4% NP-40, and protease inhibitors (MCE # HY-K0010). The extracts were then diluted to a final concentration of 20 mM HEPES (pH 7.8), 100 mM NaCl, 5% glycerol, 2.5 mM MgCl₂, 1% Triton X-100, and 0.5% NP-40 (binding buffer) for immunoprecipitation. Briefly, 10% of the whole-cell lysate was retained as an input sample, while the remaining 90% was used for immunoprecipitations. For nucleolar extracts, cell pellets were resuspended in 500 µL of SN1 buffer (20 mM HEPES-NaOH, pH 7.5; 130 mM KCl; 10 mM MgCl₂; 0.05% Igopal CA-630; 600 U/mL RNasin ribonuclease inhibitor) supplemented with cOmplete protease inhibitors (1:30 dilution). The suspension was gently mixed and centrifuged at 3,800 rpm for 3 minutes at 4°C, and the supernatant was collected for further analysis.

For semi-exogenous Co-IP, the extracts were incubated with HA (Abmart #M20031) or Flag (M2) agarose beads (Sigma) at 4°C for 4 hours. For endogenous Co-IP, the extracts were pre-cleared using protein A/G beads, followed by overnight incubation at 4°C with anti-OTUD4 (Proteintech #25070-1-AP) or anti-NPM1 (Abcam #ab15440) antibodies. Protein A/G beads (Thermo Scientific #20422) were then added, and the samples were incubated for an additional 2 hours. The beads were washed five times with IP buffer, resuspended in 4× SDS loading buffer, and boiled at 95–100°C for 5 minutes prior to Western blot analysis.

In vitro deubiquitinase assays

Ubiquitin substrates for deubiquitinase (DUB) assays were obtained via Co-IP, as described above. Reactions were conducted in enzyme buffer (50 mM Tris-HCl, pH 7.9; 150 mM NaCl; 5% glycerol; 2 mM DTT) at 37°C with the specified amounts of enzyme and ubiquitin substrates, in a total reaction volume of 50 µL for 4 hours. After the reaction, the beads were washed five times with IP buffer, resuspended in 4× SDS loading buffer, and boiled at 95–100°C for 5 minutes before Western blot analysis⁵⁹.

Mass spectrometry

Flag-vector, Flag-tagged full-length OTUD4, and its IDR3 mutant were transfected into HEK293T cells. OTUD4 protein complexes from cytoplasmic or nuclear fractions were affinity-purified using Flag (M2) agarose beads in a Co-IP assay. The immunoprecipitates were eluted three times with 150 µL of Flag peptide elution buffer (Sigma-Aldrich #FLAGIPT1). Trichloroacetic acid (Sigma-Aldrich #T0699) was added to the eluates to a final concentration of 20%, followed by incubation on ice for 1 hour. After centrifugation at 14,000 × g for 25 minutes at 4°C, the resulting pellets were washed with 500 µL of ice-cold acetone, centrifuged again, air-dried for 30 minutes in a fume hood, and stored at –20°C. Mass spectrometry was conducted with Thermo Q-Exactive/Ultime 3000 UPLC. The results are detailed in Supplementary information, Table S8.

ICD score

A total of 152 compounds inducing nucleolar stress were identified from the study by Potapova et al. Each compound was cross-verified with a valid PubChem Compound ID (CID) using the *webchem* R package. Structure data files (SDFs) for each CID were retrieved from PubChem, and immunogenic cell death (ICD) scores were computed using the *ICDPred* R package, available at <https://github.com/kroemerlab/ICDpred>. The results are detailed in Supplementary information, Table S9.

Statistics and reproducibility

Details of statistical analyses and methodologies are outlined in the figure legends, main text, or Methods section. Statistical significance was assessed using unpaired Student's *t*-tests, one-way ANOVA, two-way ANOVA, Spearman correlation, and normality tests, as appropriate. Analyses were performed using R (v4.0.2) or GraphPad Prism 9, as specified. Unless otherwise noted, data are presented as mean $\pm$ SEM, based on at least three independent experiments that yielded consistent results. Western blotting, immunofluorescence staining, immunohistochemical staining, and qPCR analyses were performed in duplicate with reproducible outcomes. Animal experiments were conducted as described in the figure legends.

In vivo data from murine models were analyzed using TumGrowth and GraphPad Prism. Kaplan–Meier survival curves were used to evaluate patient outcomes, and heterogeneity among prognostic factors was assessed using the log-rank (Mantel–Cox) test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated via univariate Cox proportional hazard regression to quantify relative risks. Pearson's correlation coefficient was used to assess correlations. Sample sizes were not predetermined using statistical methods. Following tumor inoculation, animals were randomly assigned to treatment groups; however, randomization was not applied to other experiments, and investigators were not blinded to group allocation or outcome assessments. Statistical significance was defined as $P \leq 0.05$ ($P < 0.05$, $P < 0.01$, $*P < 0.001$; ns, not significant).

Data availability

Matched pairs of RNA-seq data are available from the China National Center for Bioinformation (CNCB) under accession number CRA022375 and CRA022428. Matched pairs of ATAC-seq data are available at CNCB (CRA022403), and WGBS data are available under CNCB accession number CRA022404. H3K9me3 CUT&Tag-seq data can be accessed through CNCB (CRA022470), and H3K27ac CUT&Tag-seq data are deposited at CNCB (CRA022472). Single-cell RNA-seq datasets are publicly accessible through CNCB (CRA022599 and CRA022668). Original western blot images are included in the Supplementary Information. Raw sequencing data of CRISPR screen and processed datasets generated in this study have been deposited in the CNCB repository (CRA037447 and CRA037448). Source data are provided with this paper. Data supporting the findings of this study are available from the corresponding authors upon reasonable request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. All materials generated or analyzed during this study are included in this article and its supplementary information files. Please contact the lead contact for unique material requests. Any material that can be shared will be released via a material transfer agreement for non-commercial usage.

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ping Wang (wangp@tongji.edu.cn).

Materials availability

All materials generated or analyzed during this study are included in this article and its supplementary information files. Please contact the lead contact for unique material requests. Any material that can be shared will be released via a material transfer agreement for non-commercial usage.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Supplementary Information

This file contains Supplementary information, Fig. S1, S2 and S3. Supplementary Fig. 1: Examples of the gating strategy for FACS assays and marker gene annotations of cell clusters. Supplementary Fig. 2: Raw images of immunoblotting. Supplementary Fig. 3: Cell identities were determined using published marker gene lists. The CRISPR screen data, mass spectrometry data, sgRNA or shRNA sequence, plasmids sequence, primers sequence, ICD score data and p-value summary are provided in Supplementary information Table.

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Author contributions P.W. and L.F. conceived and supervised the study, with strategic input from Q.W., Z.G., and W.P. Experimental design was carried out by Q.W., Z.G., W.P., and P.W. Most experiments were performed and analyzed by Q.W., Z.G., and W.P. The CRISPR–Cas9 screen was conducted by Q.W., W.P., R.H., and E.T., with assistance from Z.G. and Y.L., who also contributed to the biochemical and cellular assays. Q.W., Z.G., and W.P. generated the majority of CRISPR–Cas9 knockout cell lines and performed the in vivo studies, supported by Y.W., X.Y., T.N., and P.W. Plasmid construction and mutagenesis were carried out by P.W., Z.L., and W.P. Patient-derived organoid experiments were conducted by X.Y. and Y.Y., while Z.L. and H.Z. provided samples and support for the patient-derived xenograft models. Bioinformatic analyses were performed by Q.W. and Z.G., and immunohistochemistry experiments were conducted by B.L. L.F., O.K., and G.K. provided platform resources and technical expertise. The manuscript was written by Q.W., Z.G., W.P., O.K., G.K., and P.W.

Competing interests G.K. holds patents related to immunogenic cell death (ICD) and also

serves as a scientific co-founder of Samsara Therapeutics, a company that develops novel pharmacological ICD inducers. Additionally, G.K. is a scientific co-founder of everImmune, Osasuna Therapeutics, and Therafast Bio. Furthermore, and an advisory board member for Hevolution, Institut Servier, and Rejuveron Life Sciences. All other authors declare no competing interests.

REFERENCES

1. Sreenivasan, V. K. A., Yumiceba, V. & Spielmann, M. Structural variants in the 3D genome as drivers of disease. *Nat. Rev. Genet.* <https://doi.org/10.1038/s41576-025-00862-x> (2025) doi:10.1038/s41576-025-00862-x.
2. Hafner, A. & Boettiger, A. The spatial organization of transcriptional control. *Nat. Rev. Genet.* **24**, 53–68 (2023).
3. Kresoja-Rakic, J. & Santoro, R. Nucleolus and rRNA Gene Chromatin in Early Embryo Development. *Trends Genet. TIG* **35**, 868–879 (2019).
4. Iarovaia, O. V. *et al.* Nucleolus: A Central Hub for Nuclear Functions. *Trends Cell Biol.* **29**, 647–659 (2019).
5. Rang, F. J., Kind, J. & Guerreiro, I. The role of heterochromatin in 3D genome organization during preimplantation development. *Cell Rep.* **42**, 112248 (2023).
6. Pal, M. *et al.* The establishment of nuclear organization in mouse embryos is orchestrated by multiple epigenetic pathways. *Cell* S0092-8674(25)00396-4 (2025) doi:10.1016/j.cell.2025.03.044.
7. Peng, T. *et al.* Mapping nucleolus-associated chromatin interactions using nucleolus Hi-C reveals pattern of heterochromatin interactions. *Nat. Commun.* **14**, 350 (2023).
8. Griffin, G. K. *et al.* Epigenetic silencing by SETDB1 suppresses tumour intrinsic immunogenicity. *Nature* **595**, 309–314 (2021).
9. Zhang, S.-M. *et al.* KDM5B promotes immune evasion by recruiting

SETDB1 to silence retroelements. *Nature* **598**, 682–687 (2021).

10. Falini, B., Sorcini, D., Perriello, V. M. & Sportoletti, P. Functions of the native NPM1 protein and its leukemic mutant. *Leukemia* **39**, 276–290 (2025).

11. Sharif, J. *et al.* Activation of Endogenous Retroviruses in Dnmt1(-/-) ESCs Involves Disruption of SETDB1-Mediated Repression by NP95 Binding to Hemimethylated DNA. *Cell Stem Cell* **19**, 81–94 (2016).

12. Rajshekar, S. *et al.* Hierarchical interactions between nucleolar and heterochromatin condensates are mediated by a dual-affinity protein. *Nat. Cell Biol.* **27**, 2102–2115 (2025).

13. Zhou, L. *et al.* ASB7 is a negative regulator of H3K9me3 homeostasis. *Science* eadq7408 (2025) doi:10.1126/science.adq7408.

14. Holmberg Olausson, K., Nistér, M. & Lindström, M. S. Loss of nucleolar histone chaperone NPM1 triggers rearrangement of heterochromatin and synergizes with a deficiency in DNA methyltransferase DNMT3A to drive ribosomal DNA transcription. *J. Biol. Chem.* **289**, 34601–34619 (2014).

15. Y, Z. *et al.* OTUD4 Is a Phospho-Activated K63 Deubiquitinase that Regulates MyD88-Dependent Signaling. *Mol. Cell* **69**, (2018).

16. Ren, W. *et al.* DNMT1 reads heterochromatic H4K20me3 to reinforce LINE-1 DNA methylation. *Nat. Commun.* **12**, 2490 (2021).

17. Izzo, A. *et al.* Nucleophosmin 1 cooperates with the methyltransferase DOT1L to preserve peri-nucleolar heterochromatin organization by regulating H3K27me3 levels and DNA repeats expression. *Epigenetics Chromatin* **16**, 36 (2023).

18. Swaminathan, V., Kishore, A. H., Febitha, K. K. & Kundu, T. K. Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. *Mol. Cell. Biol.* **25**, 7534–7545 (2005).

19. Lafontaine, D. L. J., Riback, J. A., Bascetin, R. & Brangwynne, C. P. The nucleolus as a multiphase liquid condensate. *Nat. Rev. Mol. Cell Biol.* **22**, 165–182 (2021).

20. Lafita-Navarro, M. C. & Conacci-Sorrell, M. Nucleolar stress: From development to cancer. *Semin. Cell Dev. Biol.* **136**, 64–74 (2023).
21. Das, R. *et al.* New roles for the de-ubiquitylating enzyme OTUD4 in an RNA-protein network and RNA granules. *J. Cell Sci.* **132**, jcs229252 (2019).
22. Mitrea, D. M. *et al.* Structural polymorphism in the N-terminal oligomerization domain of NPM1. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 4466–4471 (2014).
23. Rowe, H. M. *et al.* KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* **463**, 237–240 (2010).
24. Fang, L. *et al.* Methionine restriction promotes cGAS activation and chromatin untethering through demethylation to enhance antitumor immunity. *Cancer Cell* **41**, 1118–1133.e12 (2023).
25. Panov, K. I., Hannan, K., Hannan, R. D. & Hein, N. The Ribosomal Gene Loci-The Power behind the Throne. *Genes* **12**, 763 (2021).
26. Dopkins, N. *et al.* A field guide to endogenous retrovirus regulatory networks. *Mol. Cell* **82**, 3763–3768 (2022).
27. Dutta, S. *et al.* The crystal structure of nucleoplasmin-core: implications for histone binding and nucleosome assembly. *Mol. Cell* **8**, 841–853 (2001).
28. Li, D. *et al.* Nucleolus assembly impairment leads to two-cell transcriptional repression via NPM1-mediated PRC2 recruitment. *Nat. Struct. Mol. Biol.* **30**, 914–925 (2023).
29. Kassiotis, G. The Immunological Conundrum of Endogenous Retroelements. *Annu. Rev. Immunol.* **41**, 99–125 (2023).
30. Yang, H., Wang, H., Ren, J., Chen, Q. & Chen, Z. J. cGAS is essential for cellular senescence. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E4612–E4620 (2017).
31. Gu, Z. *et al.* Silencing of LINE-1 retrotransposons is a selective dependency of myeloid leukemia. *Nat. Genet.* **53**, 672–682 (2021).
32. Wang, J. *et al.* UV-induced somatic mutations elicit a functional T cell response in the YUMMER1.7 mouse melanoma model. *Pigment Cell Melanoma*

1770 *Res.* **30**, 428–435 (2017).

1771 33. Jhunjhunwala, S., Hammer, C. & Delamarre, L. Antigen presentation in
1772 cancer: insights into tumour immunogenicity and immune evasion. *Nat. Rev.*
1773 *Cancer* **21**, 298–312 (2021).

1774 34. Pan, H. *et al.* Immunogenic cell stress and death in the treatment of cancer.
1775 *Semin. Cell Dev. Biol.* **156**, 11–21 (2024).

1776 35. Gu, S. S. *et al.* Therapeutically Increasing MHC-I Expression Potentiates
1777 Immune Checkpoint Blockade. *Cancer Discov.* **11**, 1524–1541 (2021).

1778 36. Potapova, T. A. *et al.* Distinct states of nucleolar stress induced by
1779 anticancer drugs. *eLife* **12**, RP88799 (2023).

1780 37. Bezu, L. *et al.* eIF2 α phosphorylation is pathognomonic for immunogenic
1781 cell death. *Cell Death Differ.* **25**, 1375–1393 (2018).

1782 38. Qi, W. *et al.* NSC348884, a nucleophosmin inhibitor disrupts oligomer
1783 formation and induces apoptosis in human cancer cells. *Oncogene* **27**, 4210–4220
1784 (2008).

1785 39. Andersen, J. S. *et al.* Nucleolar proteome dynamics. *Nature* **433**, 77–83
1786 (2005).

1787 40. Stutzman, A. V. *et al.* Heterochromatic 3D genome organization is
1788 directed by HP1a- and H3K9-dependent and independent mechanisms. *Mol. Cell*
1789 **84**, 2017-2035.e6 (2024).

1790 41. Allinne, J. *et al.* Perinucleolar relocation and nucleolin as crucial
1791 events in the transcriptional activation of key genes in mantle cell lymphoma. *Blood*
1792 **123**, 2044–2053 (2014).

1793 42. Sun, W. *et al.* Histone marks enable formation of immiscible phase-
1794 separated chromatin compartments. *Cell Rep.* **44**, 116003 (2025).

1795 43. Tse, W. K. F., Jiang, Y.-J. & Wong, C. K. C. Zebrafish transforming growth
1796 factor- β -stimulated clone 22 domain 3 (TSC22D3) plays critical roles in Bmp-
1797 dependent dorsoventral patterning via two deubiquitylating enzymes Usp15 and
1798 Otud4. *Biochim. Biophys. Acta* **1830**, 4584–4593 (2013).

- 1799 44. Zhao, Y. *et al.* Noncanonical regulation of alkylation damage resistance
1800 by the OTUD4 deubiquitinase. *EMBO J.* **34**, 1687–1703 (2015).
- 1801 45. Wang, S. *et al.* Non-canonical regulation of the reactivation of an
1802 oncogenic herpesvirus by the OTUD4-USP7 deubiquitinases. *PLoS Pathog.* **20**,
1803 e1011943 (2024).
- 1804 46. M, C. *et al.* OTUD4 promotes the progression of glioblastoma by
1805 deubiquitinating CDK1 and activating MAPK signaling pathway. *Cell Death Dis.*
1806 **15**, (2024).
- 1807 47. Cui, X. *et al.* Cooperation between IRTKS and deubiquitinase OTUD4
1808 enhances the SETDB1-mediated H3K9 trimethylation that promotes tumor
1809 metastasis via suppressing E-cadherin expression. *Cancer Lett.* **575**, 216404 (2023).
- 1810 48. Margolin, D. H. *et al.* Ataxia, dementia, and hypogonadotropism caused
1811 by disordered ubiquitination. *N. Engl. J. Med.* **368**, 1992–2003 (2013).
- 1812 49. Liuyu, T. *et al.* Induction of OTUD4 by viral infection promotes antiviral
1813 responses through deubiquitinating and stabilizing MAVS. *Cell Res.* **29**, 67–79
1814 (2019).
- 1815 50. Zhu, Y. *et al.* Pharmacological suppression of the OTUD4/CD73
1816 proteolytic axis revives antitumor immunity against immune-suppressive breast
1817 cancers. *J. Clin. Invest.* **134**, e176390 (2024).
- 1818 51. Zhang, D. *et al.* Glutamine binds HSC70 to transduce signals inhibiting
1819 IFN- β -mediated immunogenic cell death. *Dev. Cell* S1534-5807(25)00117-0 (2025)
1820 doi:10.1016/j.devcel.2025.02.012.
- 1821 52. Moris, D. *et al.* Advances in the treatment of intrahepatic
1822 cholangiocarcinoma: An overview of the current and future therapeutic landscape
1823 for clinicians. *CA. Cancer J. Clin.* **73**, 198–222 (2023).
- 1824 53. Borgo, C., D’Amore, C., Sarno, S., Salvi, M. & Ruzzene, M. Protein
1825 kinase CK2: a potential therapeutic target for diverse human diseases. *Signal*
1826 *Transduct. Target. Ther.* **6**, 183 (2021).
- 1827 54. Borad, M. J. *et al.* Silmitasertib plus gemcitabine and cisplatin first-line

therapy in locally advanced/metastatic cholangiocarcinoma: A Phase 1b/2 study.
Hepatol. Baltim. Md **77**, 760–773 (2023).

55. Zhao, X. *et al.* Phosphorylation and Stabilization of PD-L1 by CK2
Suppresses Dendritic Cell Function. *Cancer Res.* **82**, 2185–2195 (2022).

56. Hashimoto, A. *et al.* Inhibition of Casein Kinase 2 Disrupts Differentiation
of Myeloid Cells in Cancer and Enhances the Efficacy of Immunotherapy in Mice.
Cancer Res. **78**, 5644–5655 (2018).

57. Humeau, J. *et al.* Inhibition of transcription by dactinomycin reveals a new
characteristic of immunogenic cell stress. *EMBO Mol. Med.* **12**, e11622 (2020).

58. Ahmad, Y., Boisvert, F.-M., Gregor, P., Cobley, A. & Lamond, A. I.
NOPdb: Nucleolar Proteome Database--2008 update. *Nucleic Acids Res.* **37**, D181-
184 (2009).

59. Deng, L. *et al.* Ubiquitination of Rheb governs growth factor-induced
mTORC1 activation. *Cell Res.* **29**, 136–150 (2019).

60. Zhang, S. *et al.* Anticancer effects of ikarugamycin and astemizole
identified in a screen for stimulators of cellular immune responses. *J. Immunother.*
Cancer **11**, e006785 (2023).

61. Vertii, A. *et al.* Two contrasting classes of nucleolus-associated domains
in mouse fibroblast heterochromatin. *Genome Res.* **29**, 1235–1249 (2019).

Figure 1. Nucleolar OTUD4 ablation redistributes heterochromatin and induces nucleolar stress.

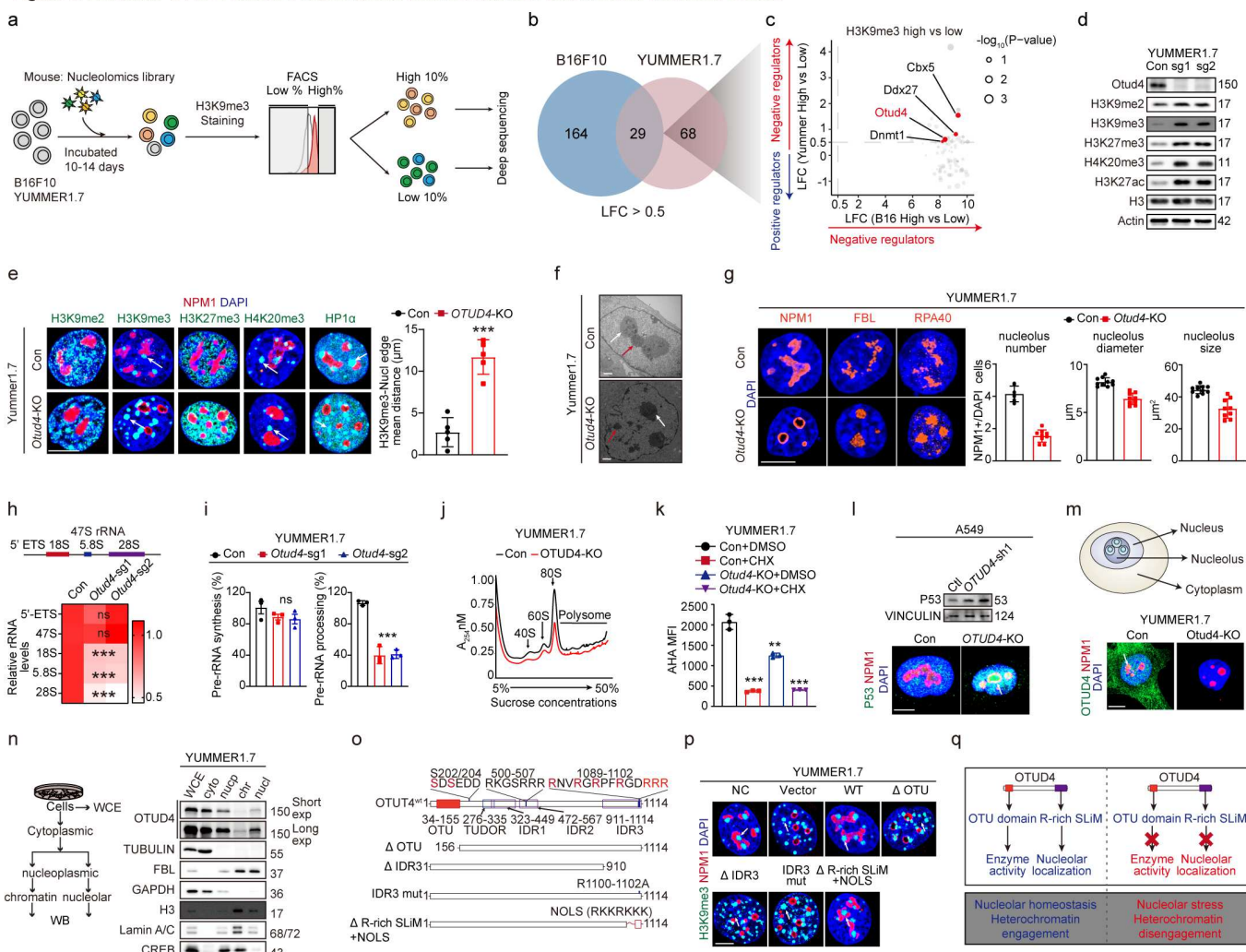
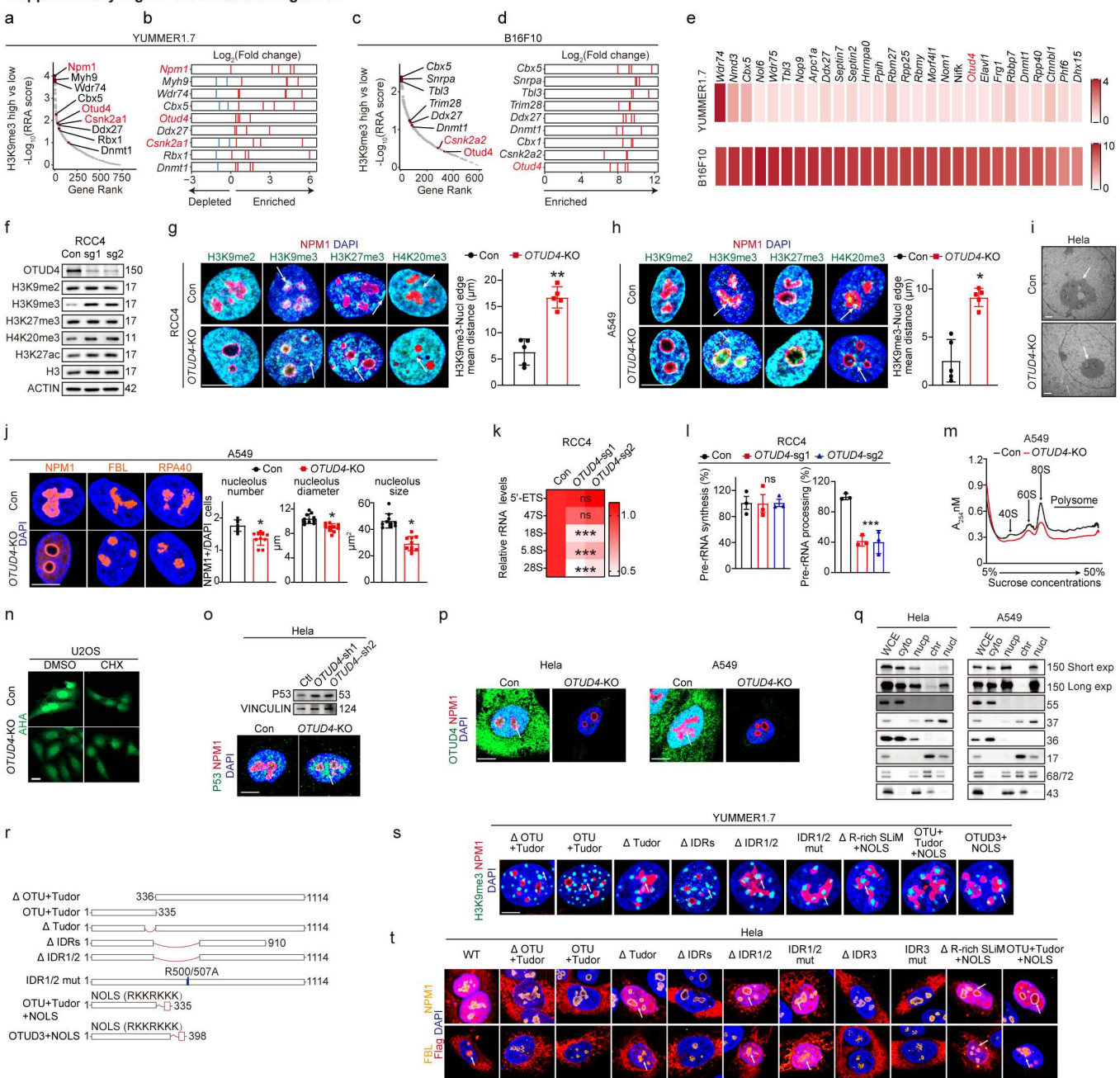


Figure 1. Nucleolar OTUD4 ablation redistributes heterochromatin and induces nucleolar stress. **a**, Workflow for identifying H3K9me3 regulators through CRISPR screens. **b**, Venn diagram of the negative regulators in two screens. LFC, log₂-fold changes. **c**, Depletion (negative ratios) or enrichment (positive ratios) of targeted genes (grey) in two screens with *Otud4*, *Cbx5* (HP1α), *Dnmt1* and *Ddx27* highlighted. **d**, Immunoblots of the indicated chromatin modifications in control or *Otud4*^{-/-} YUMMER1.7 cells. **e**, Representative immunofluorescence co-staining (left) of chromatin modifications including H3K9me2, H3K9me3, H3K27me3, H3K27ac, HP1α (green) and NPM1 (red) in wild-type or *Otud4*-ablated YUMMER1.7 cells. Quantification (right) of H3K9me3-Nucl edge mean distance (μm) in the indicated cell lines. Scale bar = 10 μm. **f**, Transmission electron micrographs showing nuclear and nucleolar morphology in control and *Otud4*-deficient YUMMER1.7 cells. Nucleoli (white) and heterochromatin (red) are indicated. Scale bar = 0.5 μm. **g**, Immunostaining (left) of nucleolar markers NPM1, Fibrillarin (FBL), and RPA40 in control and *Otud4*-deficient YUMMER1.7 cells. Scale bar = 10 μm. Quantification (right) of nucleolus number, diameter, and size in the indicated cell lines. Nucleoli were identified by NPM1 staining and manually calculated. **h**, qRT-PCR analysis of pre-rRNA and mature rRNA expression in the indicated cell lines. Values are normalized to the control group (n = 3). **i**, Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in control and *Otud4*^{-/-} YUMMER1.7 cells. **j**, Polysome profiling of control and *Otud4*^{-/-} YUMMER1.7 cells analyzed using sucrose density gradient ultracentrifugation. **k**, Bar plots showing quantification of AHA-labeled MFI by flow cytometry in control and *Otud4*^{-/-} YUMMER1.7 cells with or without CHX treatment. **l**, Immunoblots (up) of P53 expression in the indicated cell lines. Representative immunofluorescence co-staining (down) of P53 (green) and NPM1 (red) in the indicated cell lines. Scale bar = 10 μm. **m**, Representative immunofluorescence co-staining of OTUD4 (green) and NPM1 (red) in wild-type and *OTUD4*-ablated cells. Scale bar = 10 μm. **n**, Workflow (left) and immunoblot analysis (right) of OTUD4 in whole-cell extract (WCE), cytoplasmic (cyto), nucleoplasmic (nucp), chromatin (chr), and nucleolar (nucl) fractions of the indicated cells. **o**, Schematic illustration of the domain organization of wild-type (WT) OTUD4 and its truncations used in this study. Δ represents depletion, mut represents mutation, NOLS represents nucleolar localization signal, R-rich SLiM represents short arginine-rich linear motif. **p**, Representative immunofluorescence staining of NPM1 (red) or H3K9me3 (green) in control or *Otud4*^{-/-} YUMMER1.7 cells overexpressing the indicated truncations or mutations. Scale bar = 10 μm. **q**, Schematic illustration of deubiquitinase activity and nucleolar localization of OTUD4 essential for nucleolar homeostasis and perinucleolar heterochromatin distribution. Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using Two-sided Student's t-test or two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control.

Supplementary Figure S1. Related to Figure 1.



Supplementary Figure S1. Related to Figure 1. **a-e.** Nucleomics-targeted CRISPR screening reveals novel H3K9me3 regulators in YUMMER1.7 (**a**, **b**) and B16F10 (**c**, **d**). Ranked dot plots (**a**, **c**) depict the enrichment of genes in the H3K9me3-high subpopulation compared to the H3K9me3-low subpopulation. Frequency histograms (**b**, **d**) of log₂ fold changes (LFC) from the nucleomics-related CRISPR screen. Heatmaps (**e**) showing the LFCs of the H3K9me3 negative regulators in the indicated screens. **f.** Immunoblots of the indicated chromatin modifications in wild-type or *OTUD4*-ablated RCC4. **g, h.** Representative immunofluorescence co-staining (left) of chromatin modifications including H3K9me2, H3K9me3, H3K27me3, H3K27ac (green) and NPM1 (red) in wild-type or *OTUD4*-ablated RCC4 (**g**) and A549 cells (**h**). Quantification (right) of H3K9me3-Nucl edge mean distance (μm) in the indicated cell lines. Scale bar = 10 μm. **i.** Transmission electron micrographs showing nuclear and nucleolar morphology in control and *OTUD4*-deficient HeLa cells. Nucleoli (white) are marked as indicated. Scale bar = 0.5 μm. **j.** Immunostaining (left) of nucleolar markers NPM1, FBL, and RPA40 in control and *OTUD4*-deficient A549 cells. Scale bar = 10 μm. Quantification (right) of nucleolar number, diameter, and size in the indicated cell lines. Nucleoli were identified by NPM1 staining and manually quantified. **k.** qRT-PCR analysis of pre-rRNA and mature rRNA expression in control and *OTUD4*^{-/-} RCC4 cells (n = 3). **l.** Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in control and *OTUD4*^{-/-} RCC4 cells. **m.** Polysome profiling of control and *OTUD4*^{-/-} A549 cells analyzed using sucrose density gradient ultracentrifugation. **n.** Representative AHA-labeled immunofluorescence staining of sgCon or sg*OTUD4* U2OS cells with or without CHX treatment. Scale bar = 10 μm. **o.** Immunoblots (up) of P53 expression in the indicated cell lines. Representative immunofluorescence co-staining (down) of P53 (green) and NPM1 (red) in the indicated cell lines. Scale bar = 10 μm. **p.** Representative immunofluorescence co-staining of OTUD4 (green) and NPM1 (red) in wild-type and *OTUD4*-ablated cells. Scale bar = 10 μm. **q.** Immunoblot analysis of OTUD4 in whole-cell extract (WCE), cytoplasmic (cyto), nucleoplasmic (nucp), chromatin (chr), and nucleolar (nucd) fractions of the indicated cells. **r.** Schematic illustration of the domain organization of wild-type (WT) OTUD4 and its truncations used in this study. Δ represents depletion, mut represents mutation, NOLS represents nucleolar localization signal, R-rich SLIM represents short arginine-rich linear motif. **s.** Representative immunofluorescence staining of NPM1 (red) or H3K9me3 (green) in control or *OTUD4*^{-/-} YUMMER1.7 cells overexpressing the indicated truncations or mutations. Scale bar = 10 μm. **t.** Representative immunofluorescence staining of NPM1 or FBL (orange) in *OTUD4*^{-/-} HeLa cells overexpressing the indicated truncations or mutations fused with Flag (red). Scale bar = 10 μm. Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using Two-sided Student's t-test or two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control.

Figure 2. Phosphorylated OTUD4 stabilizes NPM1 oligomerization to anchor perinuclear heterochromatin.

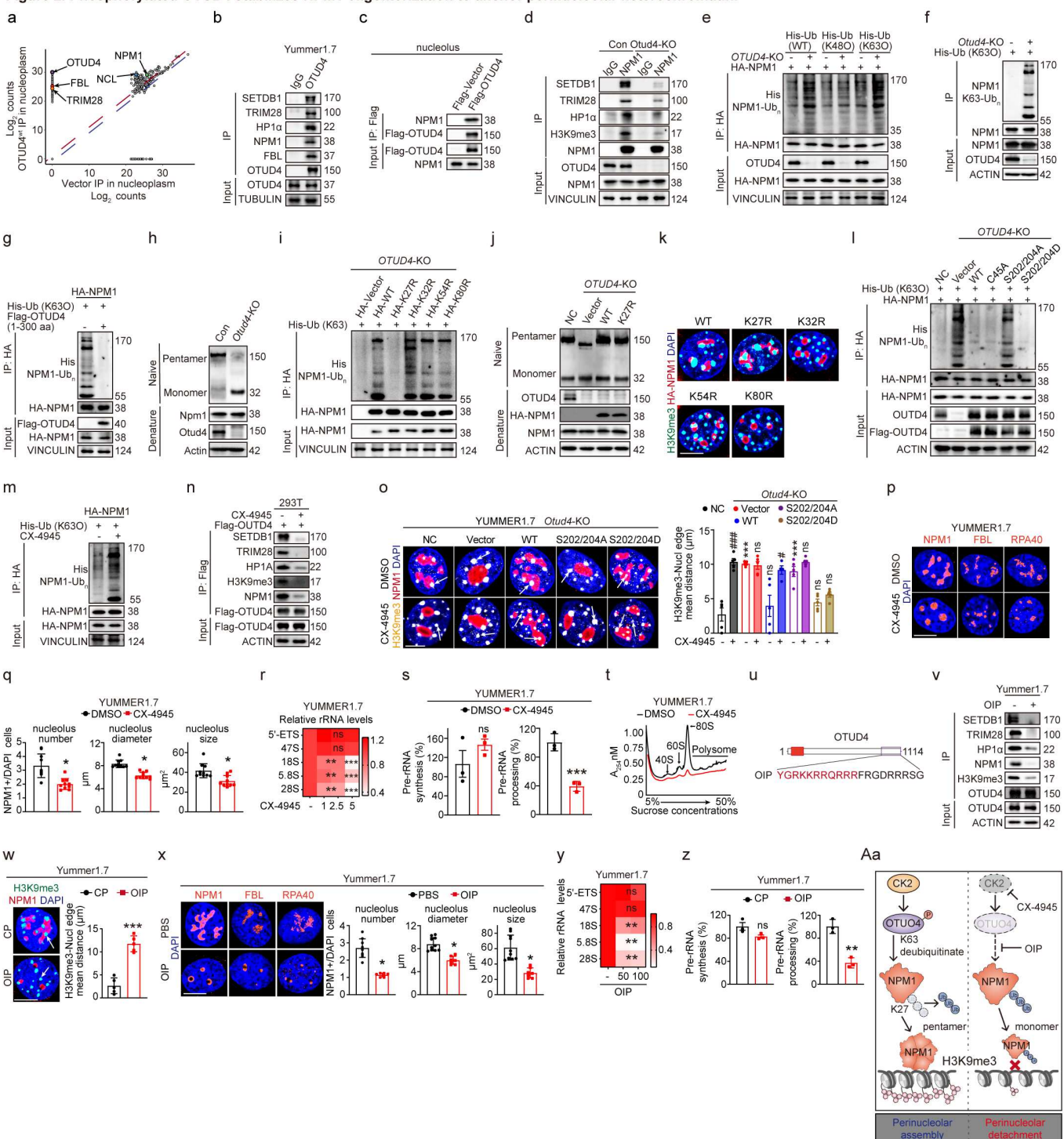
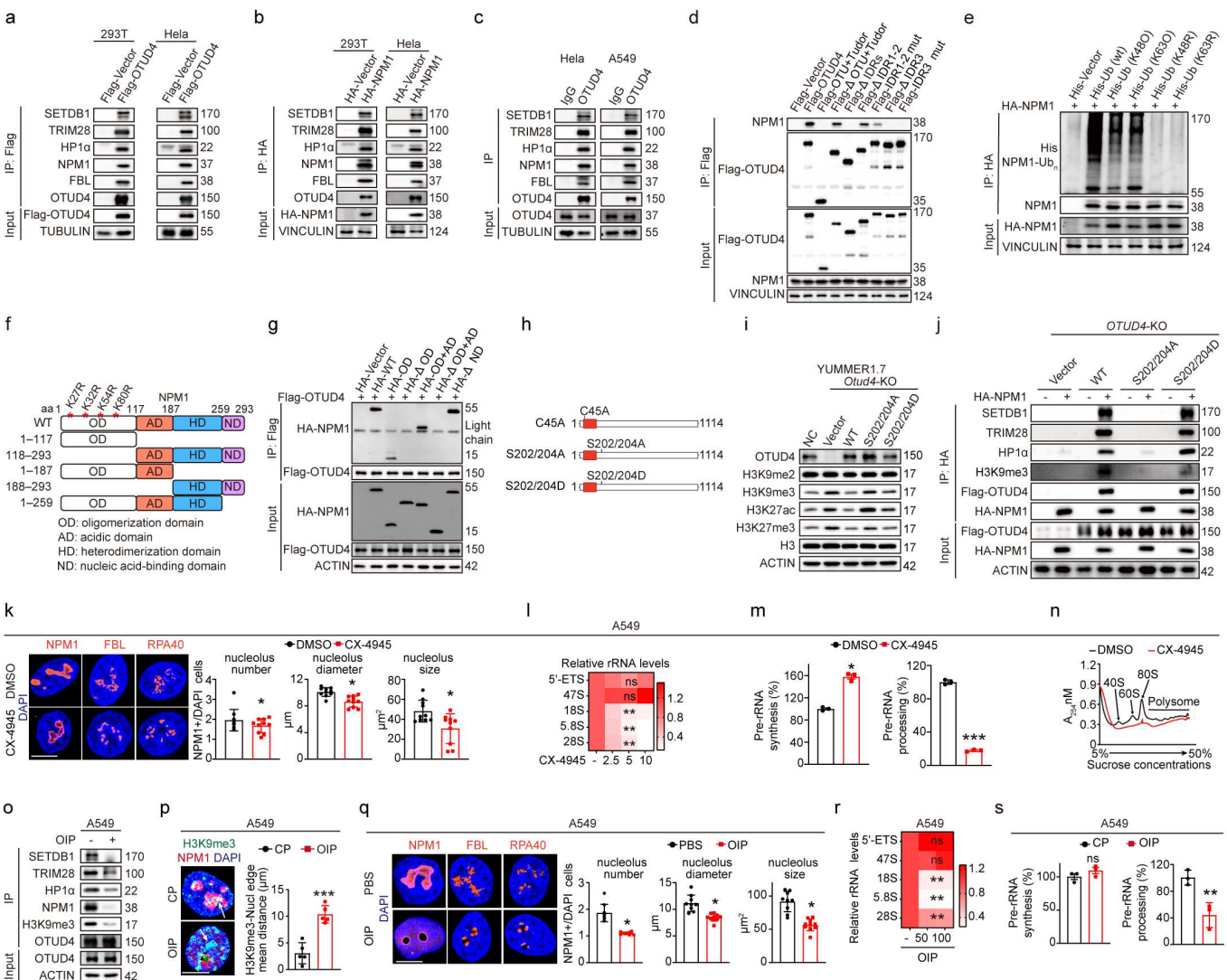


Figure 2. Phosphorylated OTUD4 stabilizes NPM1 oligomerization to anchor perinuclear heterochromatin. **a.** IP-mass analysis of OTUD4-binding proteins in the nuclear fraction. Nucleolin, NPM1; Fibrillarin, FBL; Nucleolin, NCL; Tripartite Motif Containing 28, TRIM28. **b.** Endogenous immunoprecipitation performed from YUMMER1.7 cells using control IgG and OTUD4 antibody. **c.** Co-immunoprecipitation of Flag-OTUD4 with HA-NPM1 in nuclear fractions. **d.** Endogenous immunoprecipitation performed using control IgG and NPM1 antibody in control or *Otud4*^{-/-} YUMMER1.7 cells. **e.** HA-NPM1 and His-Ub wild-type were expressed in control or *OTUD4*^{-/-} HEK293 cells, HA-immunoprecipitated after SDS denaturation, and blotted as indicated. **f.** Endogenous immunoprecipitation performed in control or *OTUD4*-deleted HEK293 cells using control IgG and NPM1 antibody. **g.** OTUD4 directly removes K63-linked ubiquitin from NPM1 in vitro. **h.** Image of native PAGE with anti-NPM1 showing oligomeric and monomeric NPM1 proteins in control or *OTUD4*^{-/-} HEK293 cells overexpressing empty vector control, wild-type NPM1 or mutants. **i.** HA-NPM1 wild-type or mutants were transfected into *OTUD4*-deleted HEK293 cells, HA-immunoprecipitated after SDS denaturation, and blotted as shown. **j.** Image of native PAGE with anti-NPM1 showing oligomeric and monomeric NPM1 proteins in control or *OTUD4*^{-/-} HEK293T cells overexpressing empty vector control, wild-type NPM1 or mutants. **k.** Representative immunofluorescence staining of HA-NPM1 (red) or H3K9me3 (green) in control or *Otud4*^{-/-} YUMMER1.7 cells overexpressing the indicated truncations or mutations. Scale bar = 10 μm. **l.** HA-NPM1 and His-Ub (K630) as well as empty vector control, wild-type OTUD4, or OTUD4 mutants were expressed in *OTUD4*^{-/-} HEK293 cells, HA-immunoprecipitated after SDS denaturation, and blotted as shown. **m.** HA-tagged Co-IP of YUMMER1.7 cells overexpressing HA-tagged NPM1 and His-Ub (K630), and then treated with or without CX-4945 (10 μM) for 24 h. HA-immunoprecipitated after SDS denaturation, and blotted as shown. **n.** Flag-tagged Co-IP of HEK293 cells overexpressing Flag-tagged OTUD4 full length, and then treated with or without CX-4945 (10 μM) for 24 h. Flag-immunoprecipitated after SDS denaturation, and blotted as shown. **o.** Control or *Otud4*^{-/-} YUMMER1.7 cells overexpressing empty vector control, wild-type OTUD4 or OTUD4 mutants as indicated, and then treated with or without CX-4945 (10 μM) for 24 h. Immunofluorescence staining (left) and bar plots (right) of H3K9me3 (orange) and NPM1 (red) in indicated cells. Scale bar = 10 μm. **p.** Immunostaining (p) of the nucleolus marker NPM1, FBL, and RPA40 in YUMMER1.7 cells treated with or without CX-4945 (10 μM) for 24 h. The quantification of nucleolus number, nucleolar diameter, and size in the indicated cell lines (q). The nucleoli were labeled by NPM1 and quantified manually. **r.** qRT-PCR analysis of pre-rRNA and mature rRNA expression in YUMMER1.7 cells treated with or without CX-4945 (10 μM) for 24 h. **s.** Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in YUMMER1.7 cells treated with or without CX-4945 (10 μM) for 24 h. **t.** Polysome profiling in YUMMER1.7 cells treated with or without CX-4945 (10 μM) for 24 h. **u.** OTUD4-interfering peptide (OIP) designed to block the OTUD4-NPM1 interaction. The TAT membrane-penetrating peptide sequence is in red. **v.** Endogenous immunoprecipitation performed from YUMMER1.7 cells using OTUD4 antibody treated with PBS or OIP (100 μM) for 48 h. **w.** Representative immunofluorescence co-staining (left) and barplot (right) of H3K9me3 (green) and NPM1 (red) in YUMMER1.7 cells treated with PBS or OIP (100 μM) for 48 h. Scale bar = 10 μm. **x.** Immunostaining (left) of nucleolar markers NPM1, Fibrillarin (FBL), and RPA40 in YUMMER1.7 cells treated with PBS or OIP (100 μM) for 48 h. Scale bar = 10 μm. Quantification (right) of nucleolar number, diameter, and size in the indicated cell lines. Nucleoli were identified by NPM1 staining and manually calculated. **y.** qRT-PCR analysis of pre-rRNA and mature rRNA expression in the indicated cell lines treated with control peptide (CP) or OIP (100 μM) for 48 h. Values are normalized to the control group (n = 3). **z.** Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in the indicated cell lines treated with CP or OIP (100 μM) for 48 h. **Aa.** Schematic model of CK2-phosphorylated OTUD4 stabilizing NPM1 oligomerization to anchor H3K9me3 heterochromatin at the nucleolar periphery. For gel source data, see Supplementary Data Figure S2. Data represent a single experiment and are presented as mean ± s.e.m. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control; #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the vector group.



Supplementary Figure S2. OTUD4 catalyses K63-linked deubiquitination of NPM1, related to Figure 1. **a. b.** Flag or HA immunoprecipitation was performed from HEK293 and HeLa cells expressing the indicated vectors and western blotted as shown. **c.** Endogenous immunoprecipitation performed from HeLa and A549 cells using control IgG and OTUD4 antibody. **d.** Flag immunoprecipitation of HEK293 cells transfected with plasmids encoding Flag-tagged OTUD4 WT or truncates. **e.** HA-NPM1 and His-Ub wild-type or mutants were expressed in HEK293 cells, HA-immunoprecipitated after SDS denaturation, and blotted as indicated. **f.** Schematic of domain organization of wild-type NPM1 and its various mutants/truncations used in this study. **g.** Flag Co-immunoprecipitation of HEK293 cells transfected with plasmids encoding Flag-tagged OTUD4 and HA-NPM1 or truncates. **h.** Schematic illustration of the mutated sites of OTUD4 used in this study. **i.** Immunoblots of the indicated chromatin modifications in control or *Otud4*^{-/-} YUMMER1.7 cells overexpressing the indicated mutations. **j.** HA-tagged co-immunoprecipitation of *OTUD4*^{-/-} HEK293 cells transfected empty vector control, wild-type OTUD4, or OTUD4 mutants as well as HA-NPM1 plasmids. **k.** Immunostaining (left) of the nucleolus marker NPM1, FBL, and RPA40 in A549 cells treated with or without CX-4945 (20 μM) for 24 h. Scale bar = 10 μm. The quantification of nucleolus number, nucleolar diameter, and size in the indicated cell lines (right). The nucleoli were labeled by NPM1 and quantified manually. **l.** qRT-PCR analysis of pre-rRNA and mature rRNA expression in A549 cells treated with or without CX-4945 (20 μM) for 24 h. **m.** Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in A549 cells treated with or without CX-4945 (20 μM) for 4 h. **n.** Polysome profiling in A549 cells treated with or without CX-4945 (20 μM) for 24 h. **o.** Endogenous immunoprecipitation performed from A549 cells using OTUD4 antibody treated with PBS or OIP (100 μM) for 48 h. **p.** Representative immunofluorescence co-staining (left) and barplot (right) of H3K9me3 (green) and NPM1 (red) in A549 cells treated with PBS or OIP (100 μM) for 48 h. Scale bar = 10 μm. **q.** Immunostaining (left) of nucleolar markers NPM1, Fibrillarin (FBL), and RPA40 in A549 cells treated with PBS or OIP (100 μM) for 48 h. Scale bar = 10 μm. Quantification (right) of nucleolar number, diameter, and size in the indicated cell lines. Nucleoli were identified by NPM1 staining and manually calculated. **r.** qRT-PCR analysis of pre-rRNA and mature rRNA expression in the indicated cell lines treated with control peptide (CP) or OIP (100 μM) for 48 h. Values are normalized to the control group (n = 3). **s.** Cell-population-based RNA pulse-chase assays were used to assess pre-rRNA synthesis (left) and processing (right) in the indicated cell lines treated with CP or OIP (100 μM) for 48 h. For gel source data, see Supplementary Data Figure S2. Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using Two-sided Student's t-test or two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control.

Figure 3. OTUD4 deficiency derepresses retroelements by relocating nucleolus-associated heterochromatin.

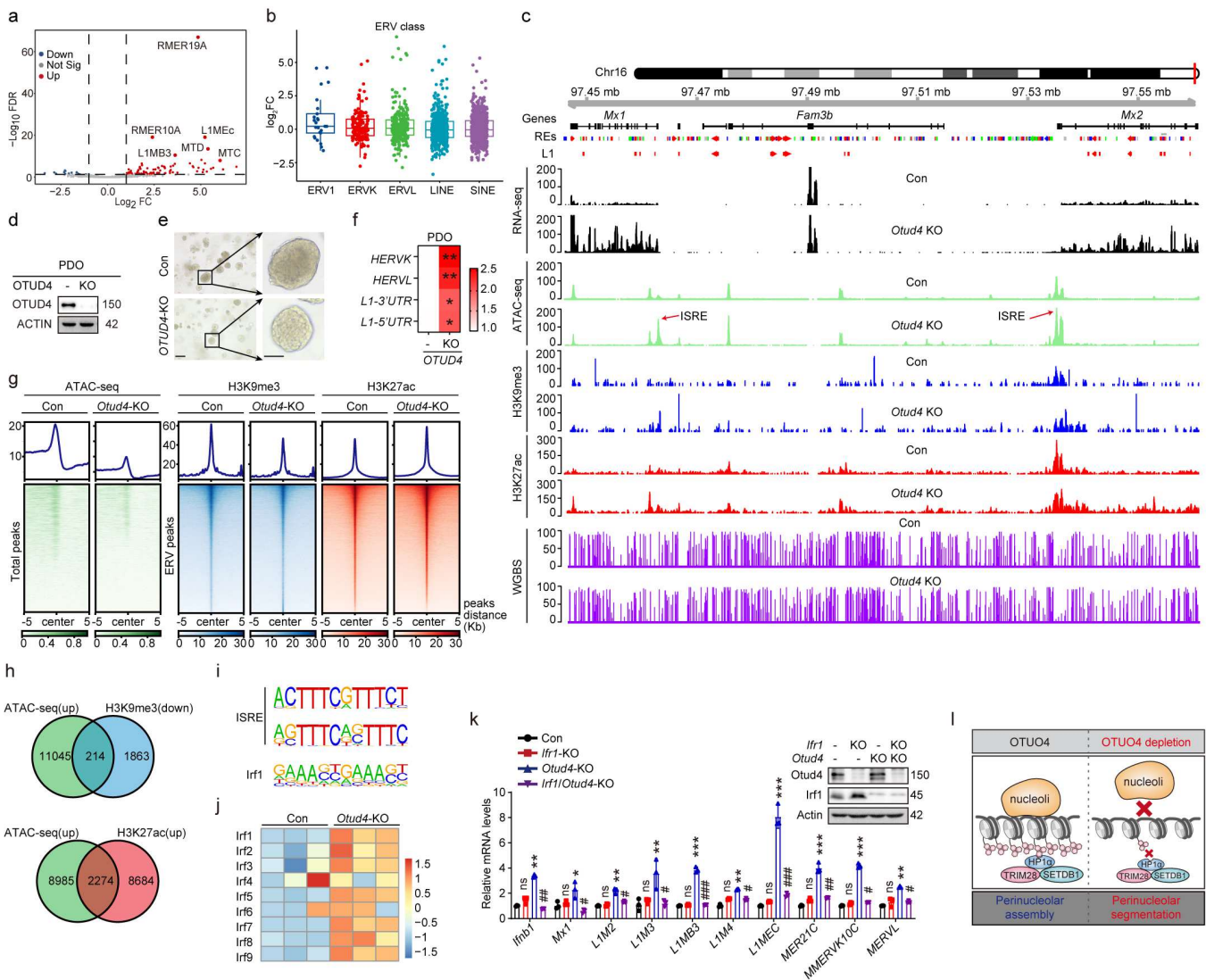
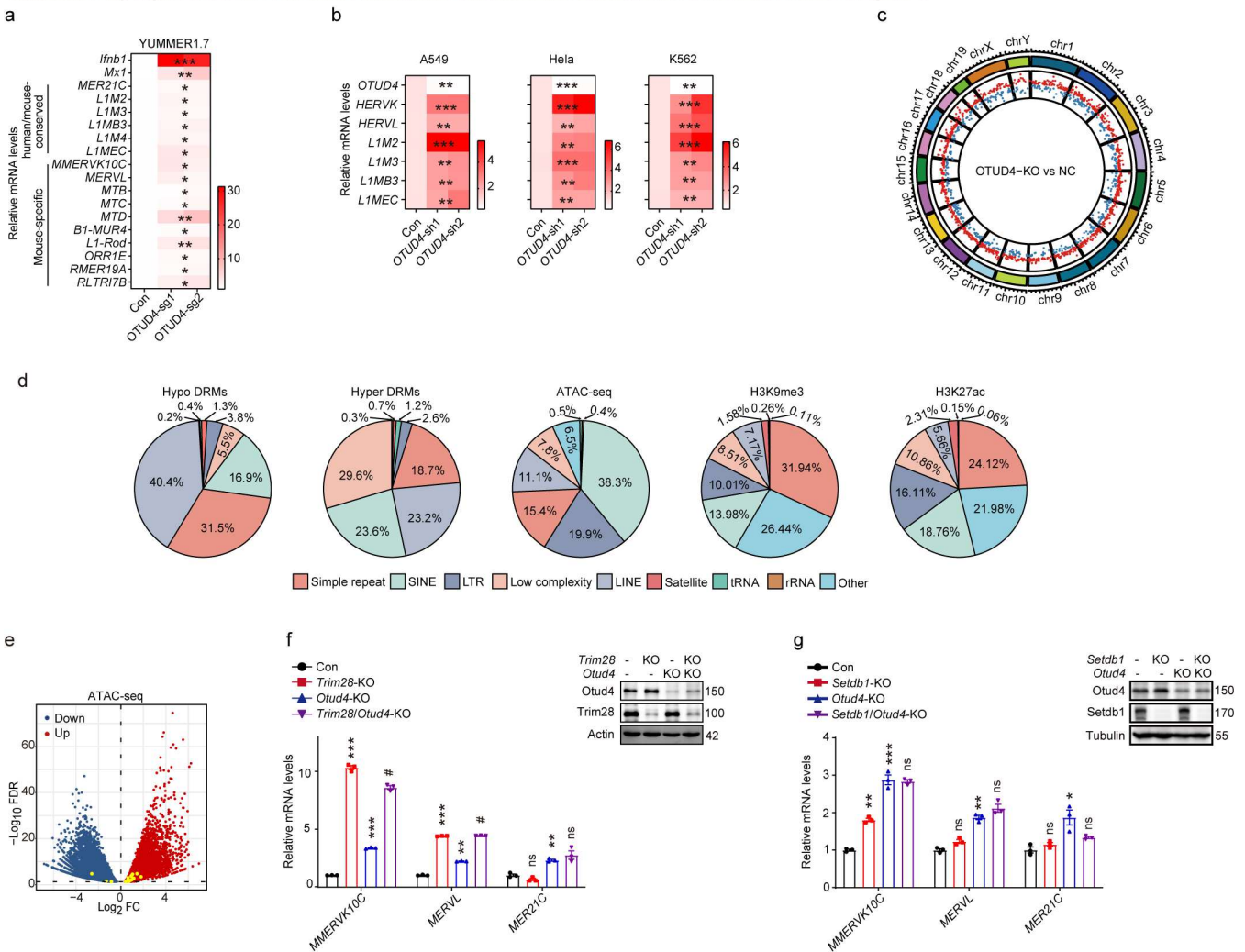


Figure 3. OTUD4 deficiency derepresses retroelements by relocating nucleolus-associated heterochromatin. **a.** Volcano plot showing retroelement loci with increased (red) and decreased (blue) expression (FDR-adjusted $P < 0.05$) in *Otud4*^{-/-} versus control YUMMER1.7 cells (n=3). The top 6 significantly differential loci are labeled. FC, fold change. **b.** Differentially expressed retroelement classes comparing *Otud4*^{-/-} versus control YUMMER1.7 cells. **c.** IGV (Integrative Genomic Viewer) screenshots of the *Mx1*, *Fam3b* and *Mx2* genes locus over a 11.5-Kb interval on chromosome 16 (Chr16), showing genomic compartments, RNA-seq, ATAC-seq, H3K9me3, H3K27ac CUT&Tag-seq and Whole Genome Bisulfite Sequencing (WGBS). For RE, blue: ERV, red: LINE1, green: SINE, gray: other. **d.** Knockout of *Otud4* in tumor organoids validated by immunoblotting. **e.** Microphotograph images of control and *OTUD4*^{-/-} patient-derived tumor organoids. Scale bar = 50 μ m. **f.** qRT-PCR analyses of the indicated RE in control and *OTUD4*^{-/-} patient-derived tumor organoids (n=3). **g.** Heatmap of whole-genome ATAC-seq, H3K9me3 and H3K27ac CUT&Tag-seq signal within RE peaks in wild-type and *Otud4*^{-/-} YUMMER1.7 cells. The RE peaks were sorted according to the different ATAC-seq signal comparing *Otud4*^{-/-} versus control YUMMER1.7 cells. **h.** Venn diagram showing overlaps of peaks for ATAC-seq, H3K9me3 or H3K27ac. **i.** Motif enrichment and sequence logos for transcription factors enriched within RE-associated ATAC-seq sites gained in *Otud4*^{-/-} YUMMER1.7 cells. IRSE, interferon stimulated response element; IRF, interferon regulatory factor. **j.** Basal expressions of IRFs in control and *Otud4*^{-/-} YUMMER1.7 cells. **k.** qRT-PCR analysis of ISG genes, MHC-IAP genes, and RE in control and *Otud4*^{-/-} YUMMER1.7 cells with or without *Irf1* depletion (n = 3). **l.** Schematic illustration of OTUD4-mediated perinucleolar H3K9me3-marked heterochromatin assembly. Data represent a single experiment and are presented as mean $\pm$ s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ compared to the OTUD4-ablated group.

Supplementary Figure S3. OTUD4 deletion redistributes nucleolus-associated heterochromatin, related to Figure 3.



Supplementary Figure S3. OTUD4 deletion redistributes nucleolus-associated heterochromatin, related to Figure 3. **a.** qRT-PCR analyses of the indicated RE in control and *Otud4*^{-/-} YUMMER1.7 cells (n=3). **b.** qRT-PCR analysis of the indicated RE in control and *OTUD4*^{-/-} A549, HeLa and K562 (n = 3). **c.** Cycle diagrams deciphering the distribution of methylation sites in whole-genome bisulfite sequencing (WGBS). red: hypermethylation; blue: hypomethylation. **d.** TE annotation in transposase-accessible chromatin with WGBS, high-throughput sequencing (ATAC-seq) peaks or H3K9me3- or H3K27ac-binding peaks in YUMMER1.7 cells (n=2). **e.** Volcano plot highlighting retroelement loci with significantly increased (red) or decreased (blue) expression (FDR-adjusted $P < 0.05$) in *Otud4*^{-/-} versus control YUMMER1.7 cells, based on ATAC-seq data. The yellow dots denote L1MEC retroelements. **f.** qRT-PCR analysis of the indicated RE in control and *Otud4*^{-/-} YUMMER1.7 cells with or without *Setdb1* depletion (n = 3). Knockout of *Otud4* and *Trim28* in tumor cells validated by immunoblotting. **g.** qRT-PCR analysis of the indicated RE in control and *Otud4*^{-/-} YUMMER1.7 cells with or without *Setdb1* depletion (n = 3). Knockout of *Otud4* and *Setdb1* in tumor cells validated by immunoblotting. Data represent a single experiment and are presented as mean $\pm$ s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ compared to the vector or OTUD4-ablated group.

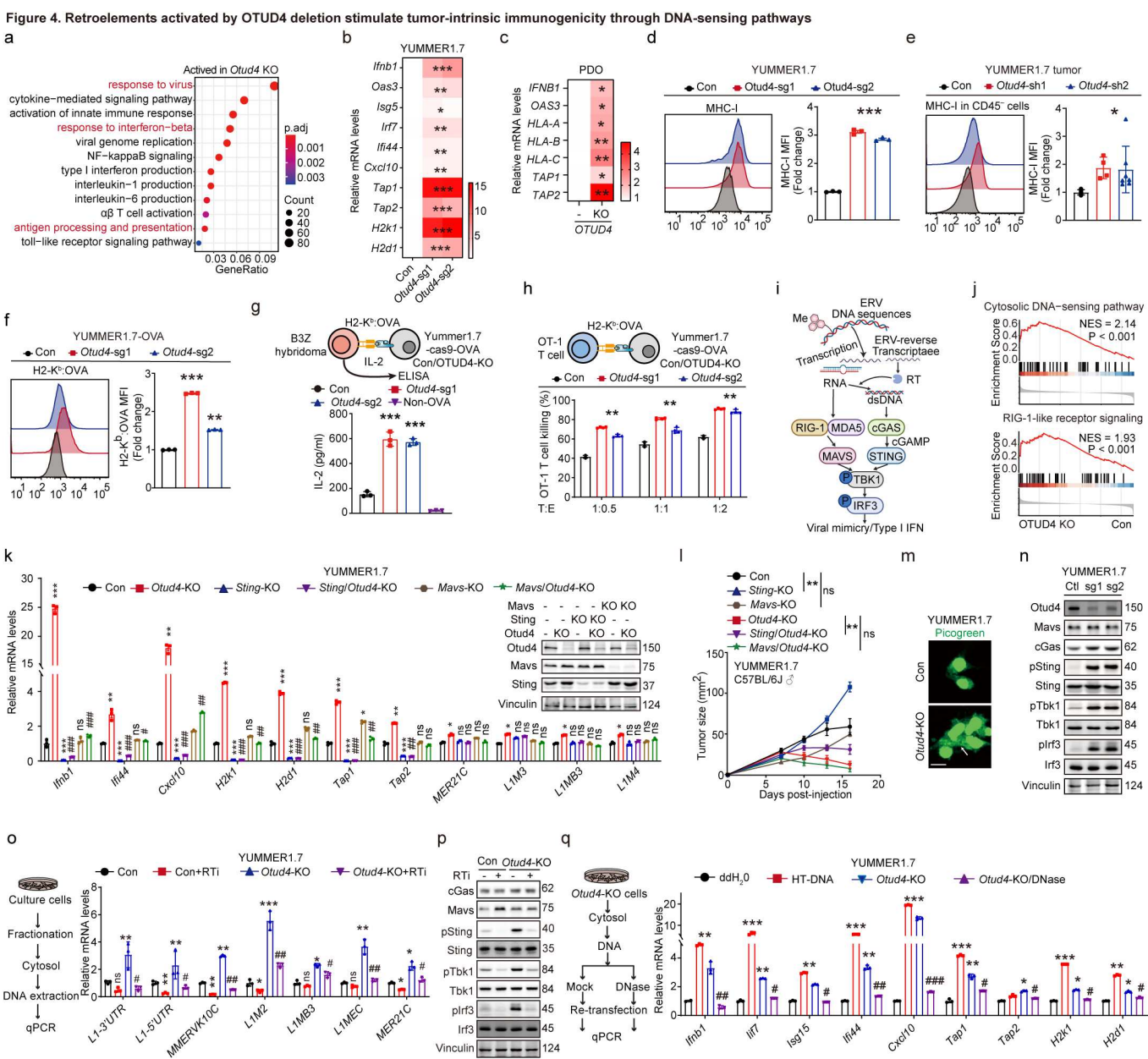
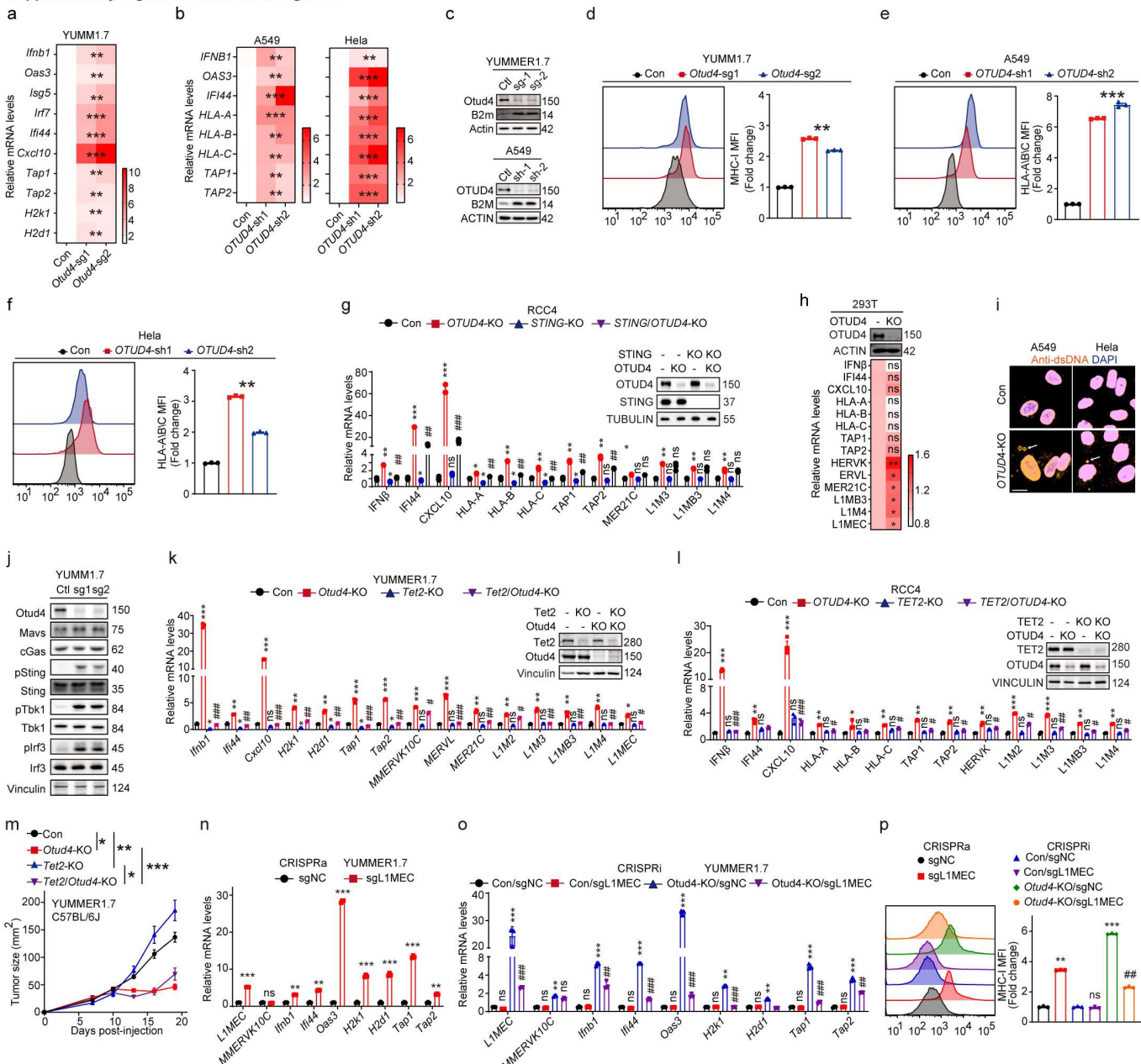


Figure 4. Retroelements activated by OTUD4 deletion stimulate tumor-intrinsic immunogenicity through DNA-sensing pathways. **a**, Pathway enrichment map of upregulated domains in *Ottd4*^{-/-} versus control YUMMER1.7 cells, derived from Gene Ontology (GO) analyses of RNA-seq data. **b**, qRT-PCR analysis of interferon-stimulated genes (ISGs) and MHC-IAP genes in control and *Ottd4*^{-/-} YUMMER1.7 cells (n = 3). **c**, qRT-PCR analysis of ISGs and HLAAP genes in control and *Ottd4*^{-/-} patient-derived organoids (n = 3). **d**, Flow cytometry analysis demonstrated increased MHC-I surface expression in *Ottd4*^{-/-} YUMMER1.7 cells compared to control group (Con). Representative histograms (left) and bar plots (right) show normalized median fluorescence intensity (MFI) values (g) (n = 3). **e**, MHC-I surface expression in tumors with *Ottd4* knockdown. Surface MHC-I expression was measured in YUMMER1.7 tumors transduced with shRNA targeting *Ottd4* or control vectors, harvested 14 days post-transplantation (n ≥ 4). **f**, Surface expression of H2-Kb: OVA in YUMMER1.7-Cas9-OVA cells. Representative histograms (left) and bar plots (right) show surface expression of H2-Kb: OVA in cells transduced with sgRNAs targeting *Ottd4* or controls (n = 3). **g**, Schematic representation of the T cell activation assay and quantification of IL-2 secretion by B3Z T cell hybridomas co-cultured with YUMMER1.7-Cas9-OVA cells transduced with sgRNAs targeting *Ottd4* or controls. (n = 3). **h**, Schematic of the T cell-mediated cytotoxicity assay and quantification of YUMMER1.7-Cas9-OVA (T) cells killed by OT-1 T cells (E), shown as a bar plot. (n = 4). **i**, Schematic illustrating the process of retroelement activation and nucleic acid-sensing pattern recognition receptor signaling. **j**, Gene set enrichment analysis of two top pathways induced by *Ottd4* deletion in YUMMER1.7 cells. FDR, false discovery rate; NES, normalized enrichment score. **k**, qRT-PCR analyses of the indicated ISGs, MHC-IAP genes, and RE in control and *Ottd4*^{-/-} YUMMER1.7 cells with or without *Sting* or *Mavs* depletion. Knockout of *Ottd4*, *Sting*, and *Mavs* in tumor cells validated by immunoblotting. **l**, Immunocompetent C57BL/6J mice were injected subcutaneously with the indicated cells. Tumor growth curves of these cells were analyzed (n ≥ 8). **m**, Control or *Ottd4*^{-/-} YUMMER1.7 cells were stained with 1 μg/mL PicoGreen (green). Scale bar = 10 μm. **n**, Western blot analyses of MAVS and DNA sensing signaling proteins. T-, total; p-, phosphorylated. For gel source data, see Supplementary Figure S2. **o**, p, Workflow (left) and qRT-PCR analyses (right) of ISGs and MHC-IAP genes in YUMMER1.7 cells transfected with cytosolic dsDNA. Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the control; #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to the OTUD4-ablated group.

Supplementary Figure S4. Related to Figure 4.



Supplementary Figure S4. Related to Figure 4. **a.** qRT-PCR analysis of interferon-stimulated genes (ISGs) and MHC-I AP genes in control and *Otd4*^{-/-} YUMMER1.7 cells (*n* = 3). **b.** qRT-PCR analysis of ISGs and HLAAP genes in A549 (left) or HeLa (right) cells transfected with control or *OTUD4*-targeted shRNA (*n* = 3). **c.** Immunoblots of B2M expression. Western blot analysis of B2M expression in control and *OTUD4*-deficient cells. **d-f.** Flow cytometry analysis of MHC-I or HLA surface expression. Representative histograms (left) and bar plots (right) depict surface expression of MHC-I in murine tumor cells YUMMER1.7 (**d**) and HLA-A/B/C in human tumor cells A549 (**e**) and HeLa (**f**). Quantification of median fluorescence intensity (MFI) is normalized to control groups (*n* = 3). **g.** qRT-PCR analyses of the indicated ISGs, HLAAP genes, and RE in control and *OTUD4*^{-/-} RCC4 cells with or without *Sting* depletion. Knockout of *OTUD4* and *STING* in tumor cells validated by immunoblotting. **h.** qRT-PCR analyses (down) of the indicated ISGs, HLAAP genes, and RE in control and *OTUD4*^{-/-} HEK293T cells. Knockout of *OTUD4* in cells validated by immunoblotting (up). **i.** Immunofluorescence staining of control or *OTUD4*-KO A549 (right) and HeLa (left) cells with anti-dsDNA antibody. Scale bar = 10 μm. **j.** Western blot analyses of MAVS and DNA sensing signaling proteins. T-, total; p-, phosphorylated. For gel source data, see Supplementary Data Figure S2. **k.** qRT-PCR analyses of the indicated ISGs, MHC-I AP genes, and RE in control and *OTUD4*^{-/-} YUMMER1.7 cells with or without *Tet2* depletion. Knockout of *Tet2* in tumor cells validated by immunoblotting. **l.** qRT-PCR analyses of the indicated ISGs, HLAAP genes, and RE in control and *OTUD4*^{-/-} RCC4 cells with or without *TET2* depletion. Knockout of *TET2* in tumor cells validated by immunoblotting. **m.** Immunocompetent C57BL/6J mice were injected subcutaneously with the indicated cells. Tumor growth curves of these cells were analyzed (*n* ≥ 6). **n.** qRT-PCR analyses of the indicated ISGs and MHC-I AP genes in YUMMER1.7 cells expressing sgNC or sgL1MEC using a CRISPRa system. **o.** qRT-PCR analyses of the indicated ISGs and MHC-I AP genes in control and *Otd4*^{-/-} YUMMER1.7 cells expressing sgNC or sgL1MEC using a CRISPRi system. **p.** Flow cytometry analysis demonstrated MHC-I surface expression in indicated cells. Representative histograms (left) and bar plots (right) show normalized median fluorescence intensity (MFI) values (*n* = 3). Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to the control; #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001 compared to the vector or *OTUD4*-ablated group.

Figure 5. OTUD4 depletion facilitates immunosurveillance.

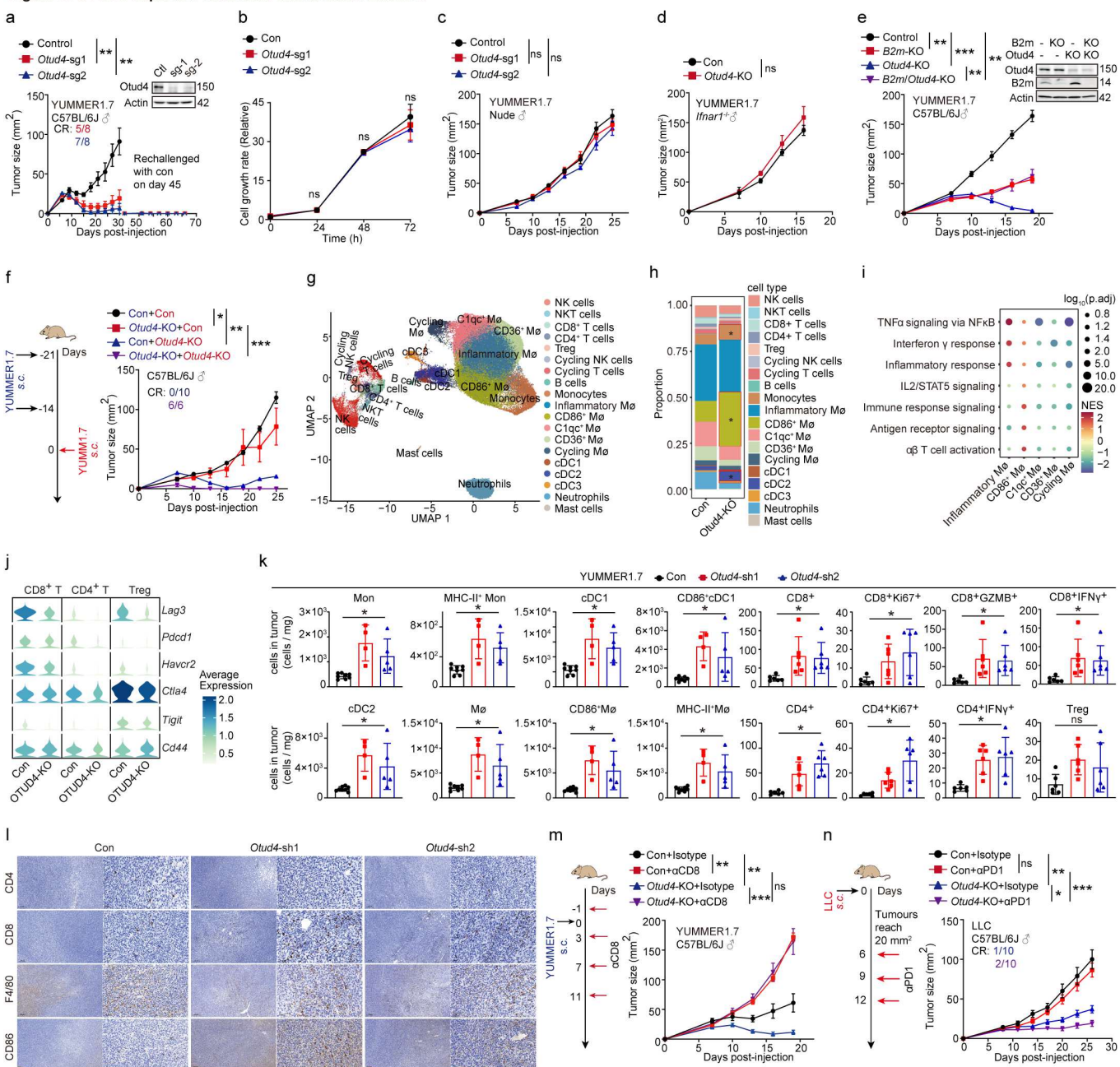
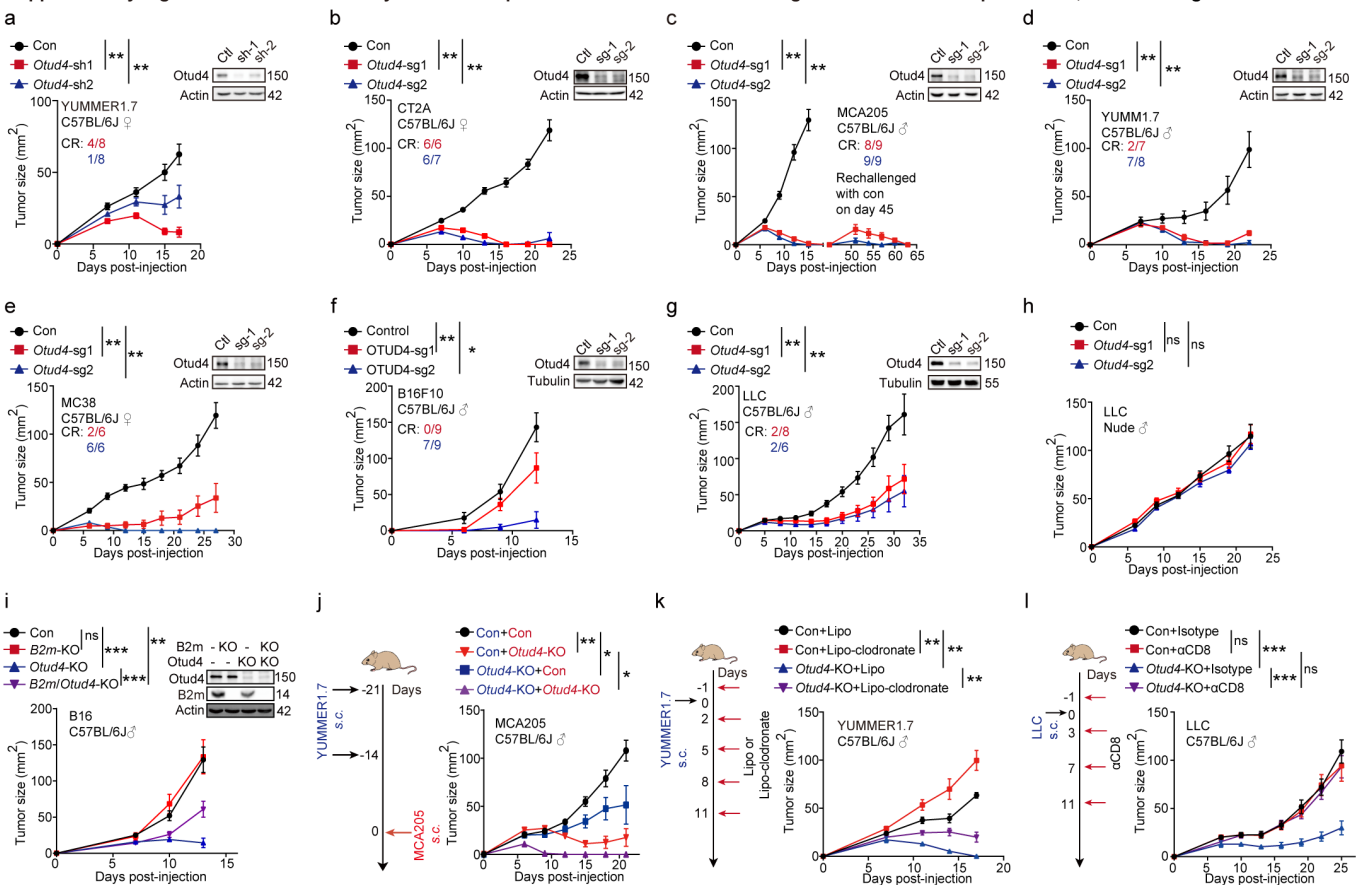


Figure 5. OTUD4 depletion facilitates immunosurveillance. **a.** Tumour growth in immunocompetent mice transplanted with the indicated YUMMER1.7 cells. *Otud4* knockout in tumor cells was validated via immunoblotting. ($n \geq 7$) **b.** Proliferation rates of wild-type and *Otud4*^{-/-} YUMMER1.7 cells, as assessed by a CCK8 assay at 24 and 48 hours. Growth rates were normalized to the control sgRNA (Con) group at 0 hours. ($n = 3$) **c.** Tumor area quantification in nude mice transplanted with control or *Otud4*^{-/-} YUMMER1.7 cells. ($n \geq 6$) **d.** Tumor growth curves of control or *Otud4*^{-/-} YUMMER1.7 cells in *Ifnar1*^{-/-} mice. ($n = 5$) **e.** Tumor area quantification in immunocompetent mice transplanted with YUMMER1.7 cells transduced with the indicated sgRNAs. Knockout of *Otud4* and *B2m* in various tumor cells validated by immunoblotting. ($n \geq 6$) **f.** Tumor growth curves in C57BL/6J mice that initially rejected control or *Otud4*^{-/-} YUMMER1.7 tumors (blue) and were subsequently rechallenged with control or *Otud4*^{-/-} YUMMER1.7 cells (red). ($n = 6$) **g.** Uniform manifold approximation and projection (UMAP) analysis of all immune cells ($n = 74,612$) identifying 19 distinct populations among CD45⁺-enriched immune cells based on scRNA-seq analysis of tumor-infiltrating leukocytes from control and *Otud4*-deficient YUMMER1.7 tumors. **h.** Relative abundances of immune cell clusters. NK, natural killer cells; Treg, regulatory T cells; Mφ, macrophages; cDCs, conventional dendritic cells. **i.** Pathway enrichment analysis of macrophage subpopulations revealed distinct functional pathways. Marker size reflects the P value, while color intensity represents the normalized enrichment score. **j.** Violin plots depicting the expression of exhaustion- and activation-associated genes within T cell clusters. **k.** Flow cytometric analysis comparing immune cell populations in control versus *Otud4*-deficient YUMMER1.7 tumors. **l.** Representative immunohistochemical staining of CD4 (CD4⁺ T cells), CD8 (CD8⁺ T cells), F4/80 (macrophages), and CD86 in control and *Otud4*-deficient YUMMER1.7 tumors 14 days post-injection. Scale bar = 100 μm. **m.** Tumor growth curves for control and *Otud4*-deficient YUMMER1.7 tumors in mice treated with anti-CD8 antibody. ($n = 10$) **n.** Tumor growth curves in C57BL/6 mice transplanted with wild-type or *Otud4*^{-/-} LLC cells treated with IgG control or anti-PD-1 antibody ($n \geq 9$). Complete response (CR) rates are reported as the number of mice with a complete response/total number of mice. Data represent one experiment. Data are mean $\pm$ s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Supplementary Figure S5. OTUD4 deficiency induces adaptive immune-mediated tumor regression across multiple models, related to Figure 5.



Supplementary Figure S5. OTUD4 deficiency induces adaptive immune-mediated tumor regression across multiple models, related to Figure 5. a-g. Tumour growth in immunocompetent mice transplanted with the indicated tumor cell lines: YUMMER1.7 (a), CT2A (b), MCA205 (c), YUMM1.7 (d), MC38 (e), B16F10 (f) and LLC (g) cells transduced with control or *Otut4*-targeting sgRNAs. *Otut4* knockdown or knockout in tumor cells was validated via immunoblotting. (n ≥ 6). Complete response (CR) rates are reported as the number of mice with a complete response/total number of mice. **h.** Tumor area quantification in nude mice transplanted with LLC cells transduced with the indicated sgRNAs. (n ≥ 6). **i.** Tumor area quantification in immunocompetent mice transplanted with B16F10 cells transduced with the indicated sgRNAs (n ≥ 6). After B16F10 cells were treated with 10 ng/mL IFNγ for 24 h, knockout of *Otut4* and B2m in various tumor cells validated by immunoblotting. **j.** Tumor growth curves in C57BL/6J mice that initially rejected control or *Otut4*^{-/-} YUMMER1.7 tumors (blue) and were subsequently rechallenged with control or *Otut4*^{-/-} MCA205 cells (red) (n ≥ 7). **k.** Tumor growth curves for control and *Otut4*-deficient YUMMER1.7 tumors in macrophage-depleted mice treated with clodronate liposomes (n = 10). **l.** Tumor growth curves for control and *Otut4*-deficient LLC tumors in mice treated with anti-CD8 antibody. (n = 10). Data represent one experiment. Data are mean ± s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. *P < 0.05, **P < 0.01 and ***P < 0.001.

Supplementary Figure S6. CK2-OTUD4-NPM1 axis mediates tumor immunogenicity, related to Figure 6.

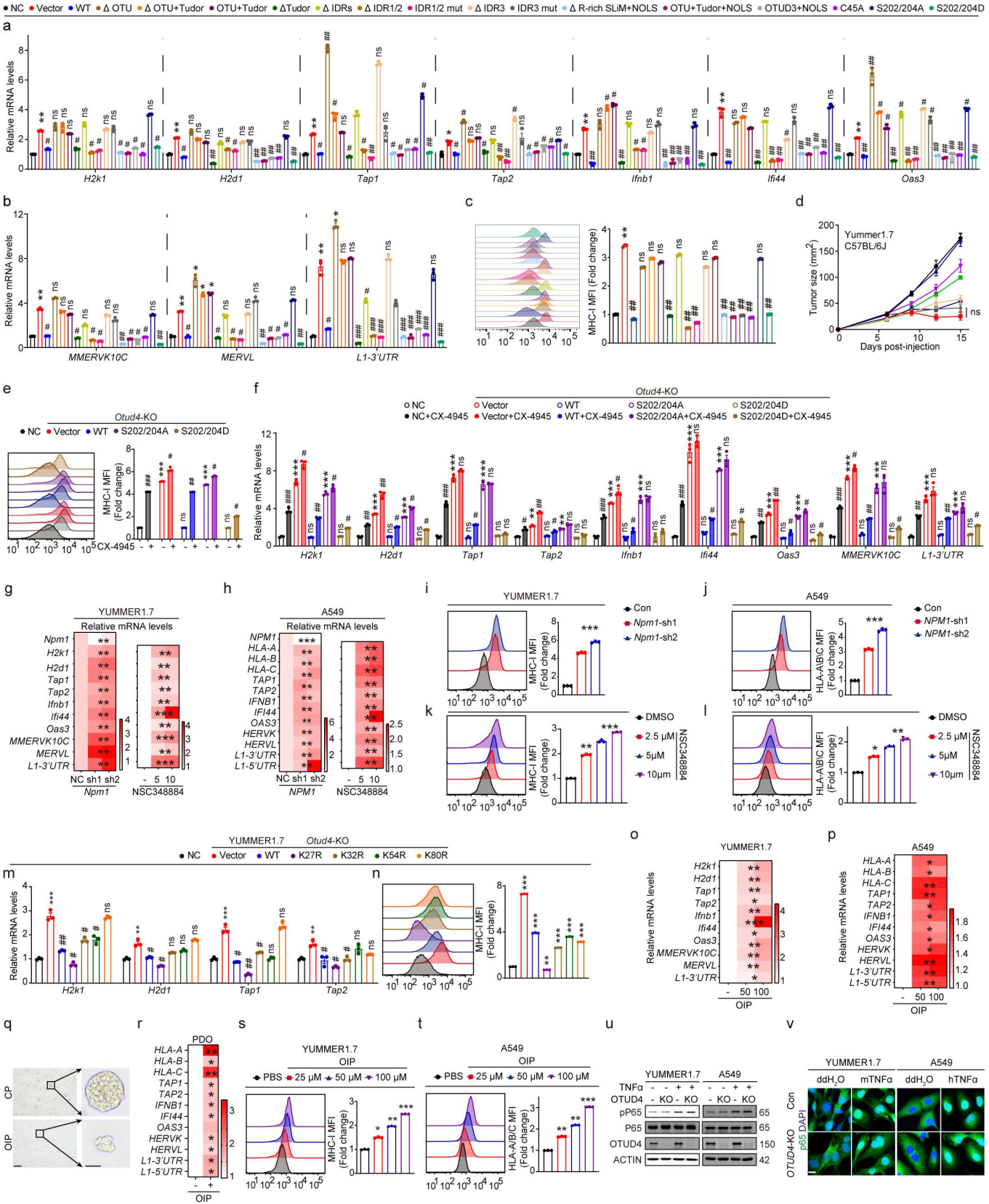


Figure 6. Targeting CK2-OTUD4-NPM1 axis augments immunogenicity.

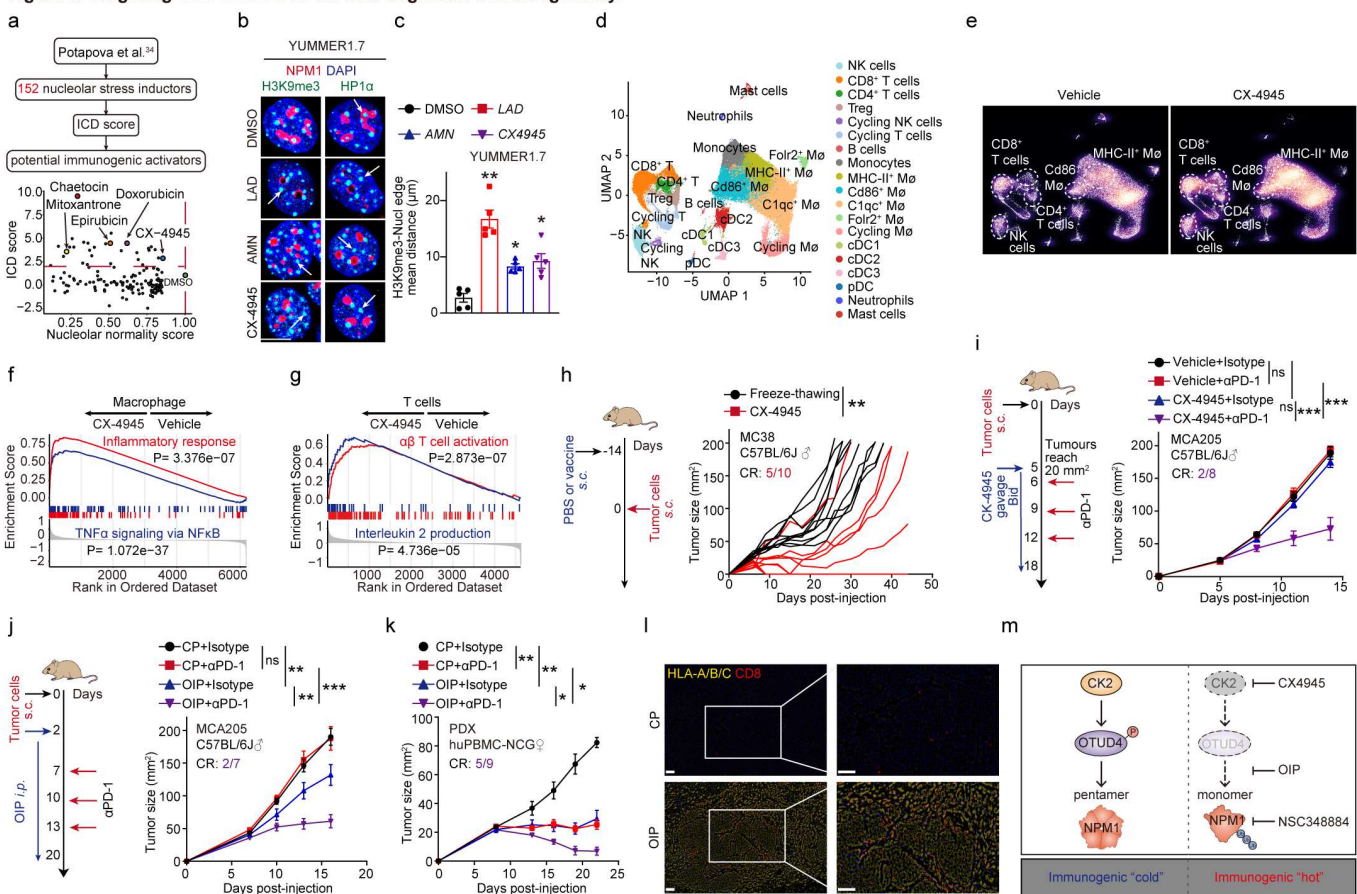
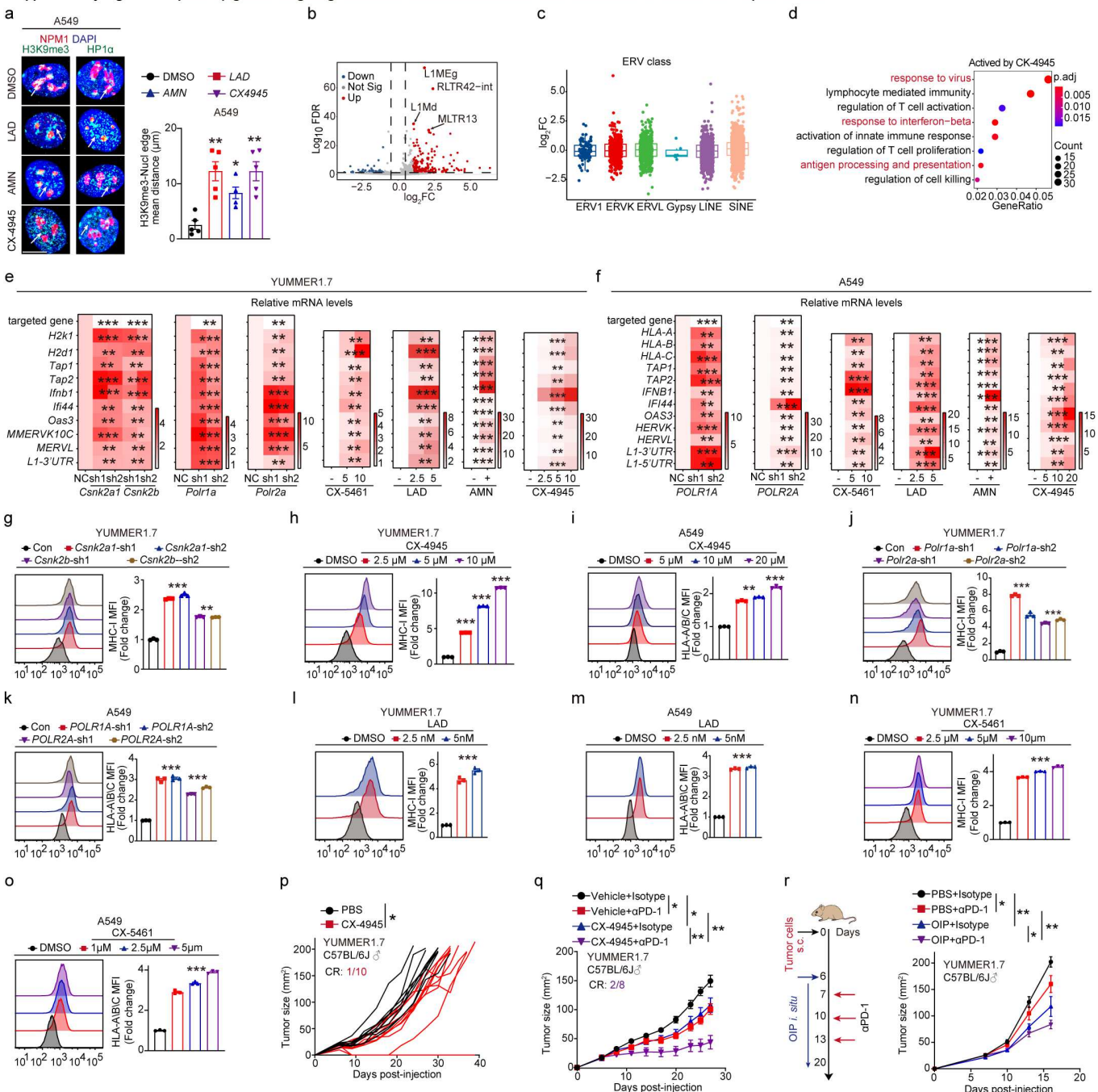


Figure 6. Targeting CK2-OTUD4-NPM1 axis augments immunogenicity. **a.** The diagram illustrates the workflow (up) and the results (down) for the library screen for nucleolar stress inducers that amplify tumor immunogenicity. **b. c.** Representative immunofluorescence (B) co-staining of chromatin modifications including H3K9me3, HP1α (green) and NPM1 (red) in YUMMER1.7 cells with or without actinomycin D (LAD, 5 nM) for 1 h, α-amanitin (AMN, 50 ng/ml) for 16 h, and CX4945 (10 μM for YUMMER1.7, 20 μM for A549) for 24 h. Bar plot are shown in (C). Scale bar = 10 μm. **d.** UMAP analysis of all immune cells (n = 55,134) with 19 unique populations identified among CD45+ enriched immune cells from scRNA-seq analysis of tumour-infiltrating leukocytes from YUMMER1.7 tumours treated with or without CX4945. **e.** Condition-specific cell density projections of cell clusters. **f. g.** Mountain plots showing enrichment scores for the relevant pathways in the macrophage cluster (f) and T cell populations (g). **h.** CX4945-mediated vaccination assay in MC38 tumors. (n = 8) **i.** Tumor growth curves in C57BL/6 mice implanted with MCA205 cells treated with CX4945 alone or in combination with anti-PD-1 antibody were plotted (n ≥ 8). **j.** Tumor growth curves in C57BL/6 mice implanted with MCA205 cells treated with OIP (10mg/kg, every other day) alone or in combination with anti-PD-1 antibody were plotted (n ≥ 6). **k.** Tumor growth curves in huPBMC-NCG mice implanted with breast cancer PDX tumors (patient #1) received control peptide (CP) or OIP (10mg/kg, every other day) alone or in combination with anti-PD-1 antibody. **l.** Immunostaining of human CD8 (red) and HLA (yellow) in PDX model treated with control peptide (CP) or OIP. Scale bar = 100 μm. Complete response (CR) was defined as the number of mice achieving a complete response relative to the total number of mice. **m.** Schematic illustration of targeting CK2-OTUD4-NPM1 axis augments immunogenicity. Data represent a single experiment and are presented as mean ± s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the DMSO group.

Supplementary Figure S7. Spatial epigenetic targeting relocalizes nucleolus-associated heterochromatin to activate RE/IFN β /MHC-I axis.



Supplementary Figure S7. Spatial epigenetic targeting relocalizes nucleolus-associated heterochromatin to activate RE/IFN β /MHC-I axis. **a.** Representative immunofluorescence (left) co-staining of chromatin modifications including H3K9me3, HP1 α (green) and NPM1 (red) in A549 cells with or without actinomycin D (LAD, 5 nM) for 1 h, α -amanitin (AMN, 50 ng/ml) for 16 h, and CX-4945 (10 μ M for YUMMER1.7, 20 μ M for A549) for 24 h. Bar plot are shown in (right). Scale bar = 10 μ m. **b.** Volcano plot showing retroelement loci with increased expression (red) and decreased expression (blue) in YUMMER1.7 cells treated with or without CX-4945 (FDR-adjusted $P < 0.05$). The top 4 significantly differential loci are labeled. FC, fold change. **c.** Differentially expressed retroelement classes in YUMMER1.7 cells treated with or without CX-4945. **d.** Gene set enrichment analysis of top pathways in YUMMER1.7 cells treated with or without CX-4945. The marker size represents the P value and the color intensity indicates the normalized enrichment score. **e-o.** YUMMER1.7 or A549 cells were knockeddown with *POLR1A*, *POLR2A* or *CK2* complex (*CSNK2A1* or *CSNK2B*). The indicated cells were treated with or without CX-4945, CX-5461, LAD or AMN as described concentrations for 24 h. **qRT-PCR** analyses of ISGs, MHC-I/HLAAP genes and ERVs were deciphered (e, f). Typical histograms (left) and bar plots (right) of MHC-I or HLA-A/B/C detected by flow cytometry in the indicated cells (g-o). MFI quantification is normalized to the Con group. **p.** CX-4945-mediated vaccination assay in YUMMER1.7 tumors. (n = 8). **q.** Tumor growth curves in C57BL/6 mice implanted with YUMMER1.7 cells treated with CX-4945 alone or in combination with anti-PD-1 antibody were plotted (n $\geq$ 8). **r.** Tumor growth curves in C57BL/6 mice implanted with YUMMER1.7 cells treated with OIP (10mg/kg, every other day) alone or in combination with anti-PD-1 antibody were plotted (n $\geq$ 6). Complete response (CR) was defined as the number of mice achieving a complete response relative to the total number of mice. Data represent a single experiment and are presented as mean $\pm$ s.e.m. Statistical significance was determined using two-way ANOVA with Benjamini-Hochberg correction. ns, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 7. OTUD4 is positively correlated with tumor progression and poor prognosis in clinical sample.

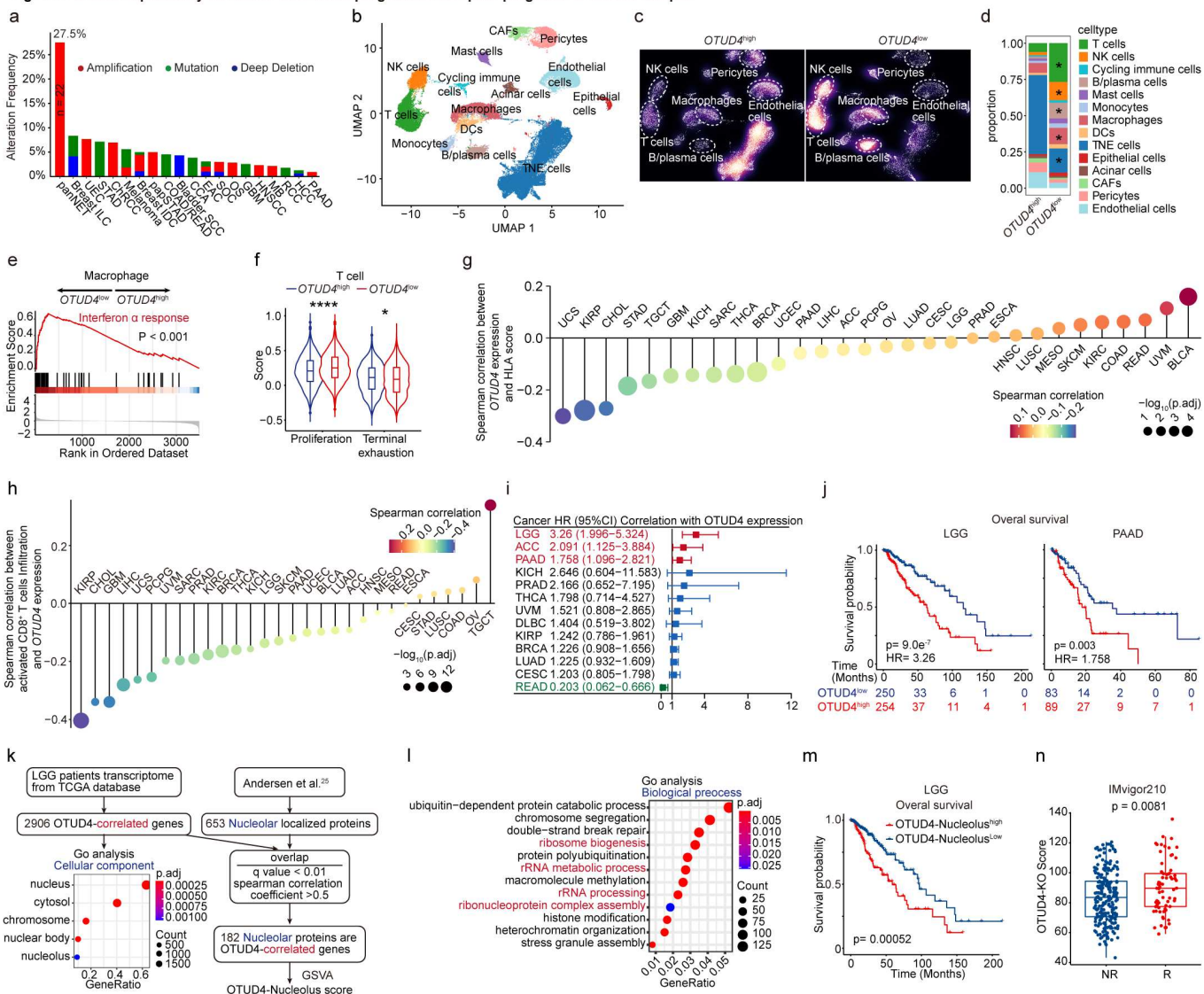
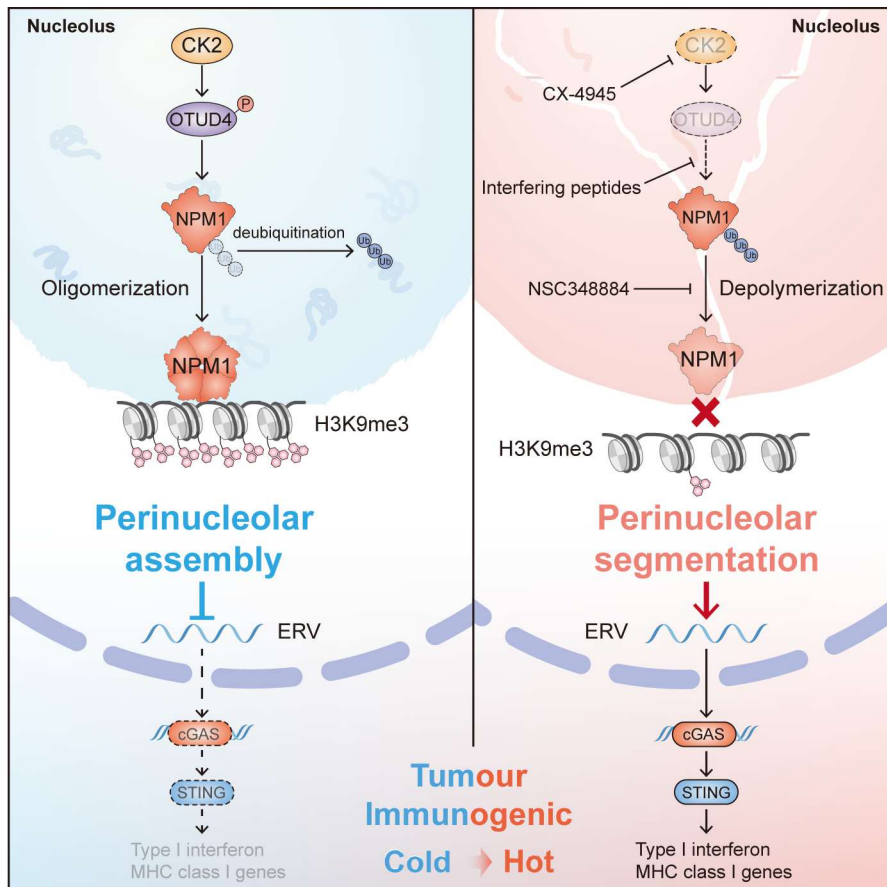


Figure 7. OTUD4 is positively correlated with tumor progression and poor prognosis in clinical sample. **a**, Distribution and types of *OTUD4* mutations identified from cBioPortal across various cancer types. Cancer types: panNET, pancreatic neuroendocrine carcinoma; ILC, invasive lobular carcinoma; UEC, uterine endometrioid carcinoma; STAD, stomach adenocarcinoma; CHRC, chromophobe renal cell carcinoma; IDC, invasive ductal carcinoma; papSTAD, papillary stomach adenocarcinoma; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; SCC, squamous cell carcinoma; CCA, cholangiocarcinoma; EAC, esophageal adenocarcinoma; SOC, serous ovarian cancer; OS, osteosarcoma; GBM, glioblastoma multiforme; HNSCC, head and neck squamous cell carcinoma; MB, medulloblastoma; RCC, renal clear cell carcinoma; HCC, hepatocellular carcinoma; PAAD, pancreatic adenocarcinoma. **b**, UMAP analysis of all cells (n = 56,628) identifying 14 distinct populations based on scRNA-seq from panNET patient samples. **c**, Condition-specific cell density projections of cell clusters. CAFs, cancer-associated fibroblasts. **d**, Relative abundances of immune cell clusters. **e**, Mountain plots showing enrichment scores for the interferon- α response gene set in the macrophage cluster. **f**, Violin plots illustrating representative proliferation and terminal exhaustion signatures in T cell populations. **g**, **h**, Pearson's correlation between *OTUD4* expression and HLA score (**g**) or activated CD8 $^{+}$ T cells infiltration (derived from the TISIDB dataset) (**h**) in TCGA cohorts. Circle size indicates statistical significance ($-\log_{10}(\text{adjusted } P \text{ value})$) of the Pearson's correlation. Blue and red indicate positive and negative values, respectively. Cancer types: KIRC, kidney renal papillary cell carcinoma; CHOL, cholangiocarcinoma; LIHC, liver hepatocellular carcinoma; UCS, uterine carcinosarcoma; PCPG, pheochromocytoma and paraganglioma; UVM, uveal melanoma; SARC, sarcoma; PRAD, prostate adenocarcinoma; KIRC, kidney renal clear cell carcinoma; BRCA, breast invasive carcinoma; THCA, thyroid carcinoma; KICH, kidney chromophobe; LGG, brain lower-grade glioma; SKCM, skin cutaneous melanoma; UCEC, uterine corpus endometrial carcinoma; BLCA, bladder urothelial carcinoma; LUAD, lung adenocarcinoma; ACC, adrenocortical carcinoma; MESO, mesothelioma; ESCA, esophageal carcinoma; CESC, cervical squamous cell carcinoma, and endocervical adenocarcinoma; LUSC, lung squamous cell carcinoma; OV, ovarian serous cystadenocarcinoma; TGCT, testicular germ cell tumors. **i**, Hazard ratios (HR) associated with *OTUD4* expression across TCGA datasets. DLBC, lymphoid neoplasm diffuse large B-cell lymphoma. **j**, Kaplan-Meier survival curves for patients with LGG (left) or PAAD (right), stratified by *OTUD4* expression levels. HR associated with *OTUD4* expression are listed; P values were calculated by log-rank test. **k-m**, Two-dimensional transcriptomic screening of the *OTUD4* network revealed associations with nucleolar-localizing proteins in LGG. Cellular components of *OTUD4*-associated genes were identified using Gene Ontology (GO) analysis (bottom). The *OTUD4*-Nucleolus score was calculated using GSVA based on the 182 nucleolar proteins correlated with *OTUD4* mRNA expression in the LGG patient transcriptome (**k**). Pathway enrichment map for *OTUD4*-associated genes in LGG derived from GO analyses (**l**). Kaplan-Meier curves showing the association between high or low *OTUD4*-Nucleolus scores and overall survival in LGG patients from the TCGA database (**m**). P values were calculated by log-rank test. **n**, Box plot illustrating *OTUD4*-KO score expression in non-responders and responders to anti-PD-1 treatment in the IMvigor210 dataset. Statistical significance was determined using Two-sided Student's t-test.



Supplementary Figure S8. Detailed model of nucleolar OTUD4-orchestrating perinucleolar heterochromatin organization mediating immunogenicity. This model delineates how OTUD4-dependent NPM1 oligomerization governs retroelement-mediated immunogenicity by modulating the spatial sequestration of perinucleolar heterochromatin. Within the nucleolus, OTUD4 interacts with NPM1, removing K63-linked ubiquitin chains to facilitate its oligomeric assembly. This process stabilizes the binding between NPM1 and H3K9me3-marked heterochromatin. Conversely, OTUD4 depletion disrupts NPM1 oligomerization to induce nucleolar stress and disengage perinucleolar H3K9me3. This heterochromatin detachment depresses retroelement, resulting in the upregulation of interferon and MHC-I antigen processing genes via DNA sensor signaling pathways, thereby augmenting tumor immunogenicity. CK2 phosphorylates OTUD4 at the S202/204 sites to specially activate its K63-linked deubiquitinating activity. CK2 inhibitor CX-4945 (①), OTUD4 depletion (②), OTUD4-interfering peptide (③), inhibition of RNA polymerase I or II (④) and NPM1 inhibitor NSC348884 (⑤) converge to instigate nucleolar stress with release of perinucleolar heterochromatin H3K9me3 for immunogenicity augmentation.

Supplementary Files

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