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Ezh2 controls cell adhesion and migration through direct methylation of the extranuclear regulatory protein talin

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A cytosolic role for the histone methyltransferase Ezh2 in regulating lymphocyte activation has been suggested, but the molecular mechanisms underpinning this extranuclear function have remained unclear. Here we found that Ezh2 regulated the integrin signaling and adhesion dynamics of neutrophils and dendritic cells (DCs). Ezh2 deficiency impaired the integrin-dependent transendothelial migration of innate leukocytes and restricted disease progression in an animal model of multiple sclerosis. Direct methylation of talin, a key regulatory molecule in cell migration, by Ezh2 disrupted the binding of talin to F-actin and thereby promoted the turnover of adhesion structures. This regulatory effect was abolished by targeted disruption of the interactions of Ezh2 with the cytoskeletal-reorganization effector Vav1. Our studies reveal an unforeseen extranuclear function for Ezh2 in regulating adhesion dynamics, with implications for leukocyte migration, immune responses and potentially pathogenic processes.

Leukocytes undergo frequent phenotypic changes to carry out immune responses and rapidly infiltrate peripheral and lymphoid tissues¹. The recruitment of circulating neutrophils and

precursors of **[Author: Correct as revised (meaning they are precursor cells that develop into DCs)? Or are they DCs that are precursors of other cells? (original ambiguous)]** dendritic cells (DCs) into inflamed tissues depends on integrin-mediated tethering and rolling of these populations on the vascular endothelium, followed by transmigration of the cells into the tissues^{2,3}. The dynamic process of migration requires the coordination of large numbers of cytosolic and transmembrane proteins whose functional activities are typically regulated by post-translational modifications that include phosphorylation, acetylation, ubiquitination and proteolytic cleavage⁴⁻⁶.

The protein with perhaps the best-defined role in regulating cell migration is talin, which directly links integrin molecules to the actin cytoskeleton. The function of talin is regulated by calpain-mediated cleavage, followed by ubiquitination or arginylation of talin⁵⁻⁷. The turnover of leukocyte focal adhesion requires proteasomal degradation of the ubiquitinated talin head domain and is antagonized by phosphorylation mediated by the cell-cycle kinase Cdk5 (ref. 6). Arginylation of talin has also been reported to be essential for cell-cell adhesion⁷, and it is possible that alternative post-translational modifications may further alter the function of talin to influence leukocyte migration.

The methylation of proteins has emerged as one of the major mechanisms by which protein function is regulated. It occurs predominantly on the side chains of constituent arginine and lysine residues but can also be found on histidine and glutamic acid and on the terminal carboxyl group of the molecule⁸. Notably, methylation of arginine is also linked to several key cytosolic and nuclear processes, including receptor signaling, protein transport and gene transcription, and may represent the most common type of protein methylation in mammalian cells⁹. In contrast, relatively few lysine-methylated proteins have been identified, and those that have been characterized are associated with transcriptional regulation¹⁰⁻¹⁵. The functional implications of lysine methylation of cytosolic proteins remain largely unclear¹⁶. Some lysine-methylated proteins in the cytosol are involved in protein translation¹⁷, and many lysine motifs that can be methylated are predicted to be prone to ubiquitination, which suggests that methylation of lysine may interfere with ubiquitin ligase activity¹⁸. It is therefore likely that methylated-lysine motifs in cytosolic proteins serve important roles in the regulation of protein biosynthesis and molecular interactions and in modifying the stability of proteins.

A potential cytosolic function has been identified for the histone methyltransferase Ezh2 in receptor-stimulated polymerization of actin¹⁹. However, the mechanism by which Ezh2 modifies extranuclear signaling processes to modulate leukocyte activity has not been defined. On the basis of that study¹⁹, we hypothesized that the unexpected cytoplasmic role of Ezh2 might have a profound effect on key leukocyte function by modulating signal-transduction pathways. Here we found that Ezh2 critically regulated leukocyte migration and adhesion dynamics via direct methylation of cytosolic talin. Ezh2-deficient innate leukocytes showed compromised migratory ability and thereby affected disease progression in mouse models of contact sensitivity and multiple sclerosis. Further molecular studies revealed that Ezh2-mediated methylation of talin1 resulted in diminished binding of talin1 to filamentous actin (F-actin) and consequently promoted the disassembly of adhesion structures in cells. In addition, targeted disruption of the interactions of Ezh2 with the cytoskeletal-reorganization effector Vav1 abolished the regulatory effect of Ezh2 on the turnover of adhesion structures. In conclusion, we suggest that talin is a cytosolic substrate of Ezh2, and we provide mechanistic insights into the influence of Vav-associated cytosolic Ezh2 on the adhesion dynamics and migratory activity of leukocytes both *in vitro* and *in vivo*.

RESULTS

Ezh2-dependent extravasation of innate leukocytes

Ezh2 exerts its epigenetic regulatory function via trimethylation of histone 3 at Lys27 (H3K27me3) and is involved in various biological and pathogenic processes^{20–22}. However, the functional importance of Ezh2 in immune responses is poorly understood. To determine the physiological function of Ezh2 in innate leukocytes, we analyzed Ezh2-deficient DCs and neutrophils in conditional *Ezh2*-null mice²³ (**Supplementary Fig. 1a**). Subsequent analyses revealed that Ezh2 was not required for the generation or distribution of neutrophils or DC subpopulations in various lymphoid and non-lymphoid organs *in vivo* (**Fig. 1**). Since H3K27me3 and its specific demethylase JMJD3-UTX are suggested to be the critical determinants of proinflammatory gene activation and inhibition of JMJD3 dampens lipopolysaccharide-induced inflammatory responses^{24,25}, we expected that Ezh2 deficiency in innate leukocytes would render mice more susceptible to autoimmune inflammatory diseases.

However, when we induced experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis^{26,27}, in *Ezh2^{f/f}Cd11c-Cre* mice, which have *Ezh2*-deficient DCs (due to deletion of *loxP*-flanked *Ezh2* alleles (*Ezh2^{f/f}*) by Cre recombinase expressed from the DC-specific promoter of *Cd11c*), the mice exhibited significantly lower disease scores than those of *Ezh2^{f/f}* mice without expression of *Cd11c-Cre* (control mice) (**Fig. 2a**, left). Flow cytometry of mononuclear cells isolated from the spinal cord of these mice at the peak of EAE progression revealed that the mice with DC-specific *Ezh2* deficiency had over 50% fewer infiltrating CD11c^{hi}MHCII⁺ DCs than the control mice had (**Fig. 2a**, right), whereas the total number of infiltrating CD45⁺ leukocytes in *Ezh2^{f/f}Cd11c-Cre* mice was similar to that in control (*Ezh2^{f/f}*) mice (**Supplementary Fig. 1b**). Immunocytochemical staining further showed that *Ezh2*-deficient DCs were more frequently co-localized with microvessels (24%) than were control cells (13%) and that *Ezh2*-deficient CD11c⁺ cells formed clusters around microvessels, whereas control (*Ezh2^{f/f}*) cells were scattered in the spinal cord parenchyma (**Fig. 2b** and **Supplementary Fig. 1b**). EAE disease progression was also not influenced by T cells activated in the periphery, because CD4⁺ OT-II T cells (which have transgenic expression of an ovalbumin-specific T cell antigen receptor) labeled with the cell tracking dye CFSE proliferated with similar kinetics in ovalbumin-immunized mice with DC-specific *Ezh2* deficiency and their *Ezh2^{f/f}* counterparts (**Fig. 2c**). This finding suggested that the attenuated EAE disease progression observed was due to the inability of *Ezh2*-deficient cells to reach the site of inflammation rather than a defect in their ability to initiate the proper adaptive immune response.

To further verify the essential role of *Ezh2* in regulating the infiltration of leukocytes into inflamed tissues, we used an ear-inflammation model to monitor neutrophil extravasation. As treatment of *Ezh2^{f/f}Mx1-Cre* mice (with deletion of *Ezh2^{f/f}* alleles by Cre expressed from the interferon-inducible gene *Mx1*) with the synthetic RNA duplex poly(I)-poly(C) induced efficient deletion of the *Ezh2^{f/f}* allele in neutrophils (**Supplementary Fig. 1c**), we induced ear inflammation by administration of croton oil to *Ezh2^{f/f}Mx1-Cre* and control (*Ezh2^{f/f}*) mice pretreated with poly(I)-poly(C). Both control and *Ezh2*-deficient neutrophils were recruited to the ear by 2 or 3 h after induction (**Fig. 2d-f**). However, a significant number of *Ezh2*-deficient neutrophils were located in microvessels and remained there even 4 h after the induction of inflammation, while a large proportion of neutrophils in the control mice had already entered the interstitial tissues by the 2-hour time point (**Fig. 2d**). Collectively, our data indicated that

Ezh2 was required for the extravasation of innate leukocytes under inflammatory conditions, which would make it an important factor in the regulation of inflammatory disease progression *in vivo*.

Ezh2 regulates integrin-dependent cell migration

To elucidate the molecular mechanisms underlying the Ezh2-regulated leukocyte migration, we generated Ezh2-deficient DCs using bone marrow cells from poly(I)-poly(C)-treated *Ezh2^{f/f}Mx1-Cre* mice. Similar to DCs observed *in vivo*, Ezh2-deficient (*Ezh2^{Δ/Δ}*) DCs derived *in vitro* from bone marrow (BMDCs) were indistinguishable from control (*Ezh2^{f/f}*) BMDCs in terms of surface-marker expression and ability to differentiate into mature DCs (**Supplementary Fig. 1d–f**). Both mature Ezh2-deficient and control DCs displayed equivalent H3K27 methylation content (**Supplementary Fig. 2a**) and similar microarray gene-expression profiles (**Supplementary Fig. 2b–d**). This result, however, was consistent with the published report that Ezh2-deficient resting T cells retain wild-type amounts of H3K27me3 and display a normal pattern of gene expression¹⁹. Since resting T cells and mature DCs exhibit only small amounts of H3K27me3, the absence of Ezh2 in these cells appeared to be compensated for by the methyltransferase Ezh1 (data not shown), which displays the same specificity for H3K27 but reportedly with lower enzymatic activity *in vitro*²⁸.

Despite their unaltered abundance of H3K27me3, Ezh2-deficient DCs and neutrophils exhibited compromised cell migration on integrin-ligand coated slides *in vitro* (**Fig. 3a,b** and **Supplementary Fig. 3a**). However, the actin polymerization and activation of downstream signaling molecules induced by the chemoattractant fMLP (N-formyl-methionine-leucine-phenylalanine) was not impaired by Ezh2 deficiency (**Supplementary Fig. 3b,c**). Mature Ezh2-deficient (*Ezh2^{Δ/Δ}*) DCs were also responsive to chemokine stimulation (with CCL19) but traversed a shorter distance than control (*Ezh2^{f/f}*) DCs did upon such stimulation (**Fig. 3a**), despite comparable expression of the chemokine receptor CCR7 (**Supplementary Fig. 3d**). Time-lapse videos generated from live-cell imaging experiments further revealed that mature control DCs were highly motile and that their dendrites rapidly attached and detached from fibronectin-coated slides (**Fig. 3c** and **Supplementary Videos 1 and 2**), while Ezh2-deficient DCs became tightly attached to the slide surface and displayed very little movement (**Fig. 3c** and

Supplementary Videos 3 and 4). These observations suggested that mature Ezh2-deficient DCs exhibited abnormal adhesion characteristics.

To more fully assess the adhesion characteristics of Ezh2-deficient DCs, we performed cell-spreading experiments on integrin ligand-coated slides. Mature Ezh2-deficient DCs rapidly attached to plates coated with the integrin ligand RGDS (a peptide epitope of fibronectin), and the number of cells that adhered to the plate was significantly higher for Ezh2-deficient (*Ezh2*^{Δ/Δ}) DCs than for control (*Ezh2*^{f/f}) cells (**Fig. 3d** and **Supplementary Fig. 4a**). Furthermore, the majority of control DCs reached maximum spread size at approximately 60 min after plating, whereas Ezh2-deficient DCs continued to spread over the plate for 2 h [**Author: Figure citation correct as added? Or is this "(data not shown)"?**] (**Fig. 3e**). In addition, the mean final surface area occupied by Ezh2-deficient DCs was more than twice that occupied by control DCs (**Fig. 3e,f**). This effect was a feature specific to the adherence of DCs to the integrin ligand-coated slides, since Ezh2-deficient DCs were similar in size to control DCs when analyzed in suspension using flow cytometry (**Fig. 3g**).

We also analyzed the expressions of surface receptors and signaling molecules in both control (*Ezh2*^{f/f}) DCs and Ezh2-deficient (*Ezh2*^{Δ/Δ}) DCs. Lack of Ezh2 did not affect the expression of the fibronectin receptors, $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins, or the non-fibronectin-responsive integrin pairs $\alpha_x\beta_2$ and $\alpha_M\beta_2$ (**Supplementary Fig. 4b**). Downstream integrin signaling molecules, including talin, FAK, paxillin, Akt, Src, Erk, PAK1 and MLC2, were also expressed at similar amounts in control and Ezh2-deficient DCs (**Supplementary Fig. 4c**). However, while integrin ligand-induced [**Author: Correct as revised? The ligands induce this activation? If not, please clarify (do not revert to) original text.**] activation of PAK1, Src and MLC2 was not affected by Ezh2 deficiency in innate leukocytes, Akt activation was greater in both Ezh2-deficient DCs and Ezh2-deficient neutrophils than in [**Author: Comparison correct as revised (here and below)? If not, it was similar in what group and what other group?**] their control counterparts, and activation of Erk1 and Erk2 was greater in Ezh2-deficient neutrophils than in control neutrophils (**Supplementary Fig. 4c,d**). Moreover, Ezh2-deficient DCs exhibited substantially larger baseline amounts of FAK phosphorylated at the autophosphorylation site Tyr397 and at the Src-dependent phosphorylation sites Tyr925 and Tyr576-Tyr577 (**Fig. 3h**), which are typically not phosphorylated in wild-type cells after stimulation with RGDS^{4,29}. These results were consistent with the rapid attachment and extensive spreading observed among

Ezh2-deficient DCs on slides coated with integrin ligands and indicated that Ezh2 regulated cell adhesion and migration through integrin-dependent pathways.

Regulation of adhesion dynamics by the Ezh2-Vav1 interaction

Since mature Ezh2-deficient DCs displayed defective migration and abnormal adhesive properties, we used immunofluorescence staining to visualize the expression of adhesion-complex components in DCs. Slowly moving cells, such as fibroblasts, form large focal adhesions that are connected to stress fibers, whereas highly migratory populations, such as mature DCs, do not normally form readily visible adhesion complexes^{30,31}. As expected, a relatively small number of mature control (*Ezh2^{f/f}*) DCs (<30%) formed visible adhesion structures, and most of them were nascent adhesions or small transient focal complexes (**Fig. 4a,b** and **Supplementary Fig. 4e**). In contrast, >40% of Ezh2-deficient (*Ezh2^{Δ/Δ}*) DCs formed frequent, enlarged focal adhesions that were connected to stress fibers (**Fig. 4a,b**, on fibronectin, and **Supplementary Fig. 4e**, on slides coated with VCAM-1, the endothelial ligand for integrin $\alpha_4\beta_1$). Ezh2-deficient innate leukocytes also displayed elevated amounts of phosphorylated tyrosine and of the signal-transduction adaptor paxillin phosphorylated at Tyr118 associated with the enlarged focal adhesions (**Supplementary Fig. 4f,g**). These characteristics resemble those of fibroblasts that exhibit compromised focal adhesions turnover due to lack of the tyrosine phosphatase SHP-2 or FAK^{32,33}.

To better determine the dynamics of adhesion-complex formation in DCs, we retrovirally transduced these cells to express green fluorescent protein (GFP)-tagged paxillin and assessed the turnover of adhesion structures by total internal reflection fluorescence microscopy. As expected, the attachment of mature control (*Ezh2^{f/f}*) DCs to fibronectin-coated slides was characterized by rapid turnover of focal complexes (15–25 min), whereas the adhesion clusters of mature Ezh2-deficient (*Ezh2^{Δ/Δ}*) DCs were enlarged and remained stable for more than 70 min (**Fig. 4c,d**). To determine whether the defective adhesion dynamics observed in Ezh2-deficient DCs were reversible, we reconstituted Ezh2-deficient DCs to express either wild-type Ezh2 or selected Ezh2 mutants via retroviral transduction. Expression of either wild-type Ezh2 or cytosolic Ezh2 restored the normal phenotype of mature DCs, these cells formed transient or no adhesion structures, whereas expression of enzymatically inactive Ezh2 or Ezh2 with diminished ability to interact with the guanine nucleotide-exchange factor Vav1

(**Supplementary Fig. 5a**) failed to reverse the adhesion abnormalities observed in Ezh2-deficient DCs (**Fig. 4e**). Collectively, our results revealed that the methyltransferase activity of Ezh2 and its interactions with Vav1 were required for the turnover of focal complexes in DCs. Because we did not observe any substantial differences in the gene-expression profile of control DCs versus that of Ezh2-deficient DCs (**Supplementary Fig. 2b–d**), we concluded that Ezh2 modified the adhesion and migration of DCs by interacting with and/or methylating molecules in the cytosol, rather than by modifying gene expression.

Ezh2 recruited by Vav1 directly methylates talin1

Vav1 is a hematopoietic-specific guanine nucleotide exchange factor that is involved in integrin signaling via interactions with talin^{34,35}, a protein that physically links integrins to the actin cytoskeleton and is essential for integrin-dependent cell adhesion and migration^{5,6,36,37}. As Ezh2 interacts with Vav1 (refs. 19,38), we hypothesized that talin links Ezh2 activity with integrin signaling. In a series of reciprocal immunoprecipitation assays, we confirmed that Ezh2 formed cytosolic complexes with Vav1 and talin1 in DCs (**Fig. 5a**). We predicted that the presence of Ezh2 in this cytosolic complex may influence the nature of the interactions of Vav1 with talin. However, the formation of Vav1-talin1 complexes was not affected by Ezh2 deficiency in mature DCs (**Fig. 5b**), and Ezh2 interacted with talin1 and with Vav1 with similar kinetics (**Fig. 5c**).

The stability of talin protein and its interactions with other cytosolic proteins are known to be regulated by post-translational modification events^{5,6,36}. Given the known methyltransferase activity of Ezh2, we hypothesized that Ezh2 is recruited by Vav1 to mediate the methylation of lysine residues of talin. To test this, we used talin1 immunoprecipitated from DCs as the substrate in a methyltransferase assay with a recombinant Ezh2 complex containing EZH2, SUZ12, EED and PHF1 and a radioactive methyl donor ([³H]S-adenosyl methionine). We found that talin1 was methylated by an active recombinant Ezh2 complex but not by an inactive recombinant Ezh2 complex (**Fig. 5d–f**). To assess the methylation of talin1 under more physiological conditions, we expressed wild-type Ezh2 or 'enzymatically hyperactive' Ezh2 mutants in HEK293T human embryonic kidney cells and assessed the methylation of immunoprecipitated endogenous talin1 with antibody to total methylated lysines. Similar overexpression of Ezh2 protein in primary hematopoietic lineage cells is not

possible due to unknown toxic effects to the cells (data not shown). As expected, expression of hyperactive Ezh2 mutants promoted methylation of talin1 in wild-type HEK293T cells (**Fig. 5g**), but in cells in which expression of Vav2 (the main member of the Vav family expressed in these cells) was reduced via knockdown mediated by short hairpin RNA, the methylation of talin1 was diminished (**Fig. 5h**). Similarly, decreased methylation of talin1 in cells expressing mutant Ezh2 with diminished ability to interact with Vav1 was also correlated with less co-localization of this mutant with focal adhesions (**Supplementary Fig. 5b,c**). These results suggested that Ezh2 mediated methylation of talin but that the recruitment of Ezh2 to talin *in vivo* required interaction with Vav proteins.

Methylation of talin1 at Lys2454 promotes cleavage of talin1

The methylation of lysine residues in non-histone proteins is commonly involved in the regulation of protein stability or protein-protein interactions^{16,39}. In particular, methylation of the transcription factor GATA-4 by Ezh2 results in the attenuation of its transcriptional activity by disrupting interactions with the histone acetyltransferase p300 (ref. 14). Ezh2-mediated methylation is also reported to destabilize the transcription factor ROR α ^{14,15}. As we did not observe substantial changes in expression amounts of talin1 protein in the absence of Ezh2 and the interaction between Vav1 and talin1 in DCs was not impaired by Ezh2 deficiency (**Fig. 5b**), Ezh2-mediated talin1 methylation must regulate turnover of focal complexes through a different mechanism. Additional *in vitro* methyltransferase assays with peptides as substrates revealed that the methylated lysine residues in talin1 were located within two regions consisting of amino acids 2440–2467 and 2506–2532, in the C-terminal actin-binding and dimerization domains⁴⁰ (**Fig. 6a**). Subsequent analysis by mass spectrometry determined that trimethylation of Lys2454 was mediated by Ezh2 (**Fig. 6b**, **Supplementary Fig. 6** and **Supplementary Tables 1 and 2**). Through spectral counting, we estimated that the extent of trimethylation of Lys2454 on *in vitro*–methylated peptide was 37% (**Supplementary Fig. 6**); however, the proportion of methylated protein *in vivo* is difficult to estimate.

Since cleavage of talin is one of the major inducers of adhesion turnover, we determined whether Ezh2-mediated methylation of talin affects the cleavage of talin. We overexpressed variants of Ezh2 in adherent MCF10a human mammary cells and lysed the cells directly on the tissue culture plate. This protocol prevents cleavages of talin1 introduced by

mechanical force during cell harvesting and is not applicable to semi-adherent mature DCs. Talin1 isolated with this protocol was mostly full length, whereas we observed increased cleavage of talin1 in cells expressing wild-type or cytosolic Ezh2 (**Fig. 6c**). Moreover, mutant talin1 with substitution of the lysine at position 2454 of with glutamine, which cannot be methylated (K2454Q), underwent less cleavage in HEK293T cells than did wild-type talin1 (**Fig. 6d**). In contrast, methyl-mimicking replacement with phenylalanine (K2454F) rendered the mutant talin1 more susceptible to cleavage than wild-type talin1 (**Fig. 6d**). In addition, treatment of HEK293T cells expressing hyperactive Ezh2 with a calpain inhibitor revealed that Ezh2-mediated trimethylation of Lys2454 occurred on full-length talin (**Fig. 6e**). We therefore concluded that Ezh2-mediated methylation of talin1 at Lys2454 promoted cleavage of talin.

Ezh2-mediated methylation of talin1 disrupts binding to F-actin

Because Lys2454 of talin1 is structurally not near the site for cleavage by calpain, Ezh2-mediated methylation of talin may indirectly promote cleavage of talin1. Notably, Lys2454 of talin1 is located within the actin-binding surface and is adjacent to two critical residues, as defined before⁴⁰ (**Fig. 7a**). Because the binding of talin to F-actin provides the mechanical force required for the maturation of focal adhesions^{41,42}, our result suggested that Ezh2-mediated methylation of talin might disrupt the binding of talin to F-actin and thereby regulate both the turnover of focal complexes and cell migration. When we overexpressed wild-type or cytosolic Ezh2 in the human mammary epithelial cell line H16N2, the disassembly of focal adhesions was greatly enhanced relative to that in cells when endogenous Ezh2 was knocked down (**Fig. 7b**). In contrast, Ezh2 mutants with diminished ability to interact with Vav1 or disrupted enzymatic activity were unable to promote the disassembly of focal adhesions in these cells (**Fig. 7b**). Expression of a cytosolic, enzymatically inactive Ezh2 mutant fused to GFP further revealed the association of Ezh2 with enlarged focal adhesions; this association was less prominent in cells expressing cytosolic Ezh2 fused to GFP (**Fig. 7c**).

We further assessed the importance of Lys2454 of talin1 in actin binding by actin co-sedimentation assays and surface plasmon resonance (SPR) studies. The efficiency with which talin underwent sedimentation together with F-actin was significantly increased when the lysine residue at position 2454 in a peptide of talin1 amino acids 2300–2541 (talin1(2300–2541)) was replaced with glutamine (K2454Q), relative to results obtained for wild-type talin1, and

binding of F-actin to the methyl-mimicking talin1(K2454F) mutant was lower than its binding to wild-type talin1 (**Fig. 7d** and **Supplementary Fig. 7a,b**). Moreover, the dissociation constant (K_d) for dissociation of the K2454Q mutant of talin1(2300–2541) from F-actin was 3.4 times lower than that calculated for wild-type talin1(2300–2541) ($P < 0.0001$), whereas the talin1(K2454F) mutant formed measureable complexes with F-actin only at higher concentrations and dissociated from F-actin at a rate similar to that calculated for wild-type talin1 ($P = 0.09$) (**Supplementary Fig. 7c**). In addition, the talin1(K2454Q) mutant and talin1(K2454F) mutant showed lower and higher affinity for binding to F-actin than an actin-binding–enhancing talin1 mutant with substitution of aspartic acid for the asparagine at position 2447 and an actin-binding–repressive talin1 mutant with substitution of valine for the aspartic acid at position 2444 (ref. 40), respectively (**Supplementary Fig. 7b**). Notably, Asp2447 is structurally very near Lys2454, and replacement of these residues with a polar uncharged amino acid (asparagine or glutamine) increased the affinity of their binding to F-actin (**Supplementary Fig. 7b**). In contrast, replacement with a hydrophobic residue (K2454F) diminished the affinity of its binding to F-actin (**Supplementary Fig. 7b**). Because trimethylation of a lysine residue does not remove the positive charge but instead increases hydrophobicity, regulation of this actin-binding pocket under physiological conditions is probably modulated by subtle changes in hydrophobic properties. Finally, we also demonstrated that the binding of talin1(2450-2460) peptide [Author: Which peptide is this? talin1(2300–2541)? Or one of the others mentioned above?] with trimethylated Lys2454 to F-actin was less than that of unmodified peptide (**Supplementary Fig. 7d**). Thus, our data provided compelling support for the hypothesis that Ezh2-mediated talin methylation interferes with binding to F-actin and hence regulates adhesion dynamics.

To assess the role of Lys2454 of talin1 in adhesion dynamics under physiological conditions, we overexpressed talin1 mutants with replacement of this residue with alanine (K2454A), glutamine (K2454Q) or phenylalanine (K2454F) in H16N2 cells. Our results showed that expression of talin1(K2454F) diminished the total number of adhesion structures and increased the frequency of cells without visible adhesion structures (**Fig. 7e,f**). In contrast, the number of focal adhesions and fibrillar adhesions in cells expressing talin1(K2454A) or talin1(K2454Q) were significantly greater than that in cells expressing wild-type talin1 (**Fig. 7e,g**), consistent with the increased actin binding seen *in vitro* (**Fig. 7d**). Moreover, expression

of the talin1(K2454F) mutant 'rescued' the excessive cell spreading and defective cell migration of Ezh2-deficient ($Ezh2^{\Delta/\Delta}$) BMDCs derived from poly(I)-poly(C)-treated $Ezh2^{f/f}$ Mx1-Cre mice, while expression of talin1(K2454Q) converted control ($Ezh2^{f/f}$) cells to Ezh2-deficient DC-like cells (**Fig. 8**). Even though overexpressed wild-type talin1 was able to modulate cell-migratory activity to some extent (on surfaces coated with VCAM-1 at a concentration of 5 μ g/ml), the specific rescue effects we obtained on surfaces coated with VCAM-1 at a concentration of 10 or 20 μ g/ml and with methyl-mimicking talin1(K2454F) mutant were clear (**Fig. 8**). Together these data demonstrated that Ezh2 directly promoted methylation of talin and functionally enhanced cellular disassembly of focal adhesions through disrupting the binding of talin to F-actin.

Ezh2 does not control integrin-independent migration

The impaired extravasation of Ezh2-deficient innate leukocytes *in vivo* probably results from migration defects similar to those we observed *in vitro*. On the basis of our *in vitro* data, we speculated that without Ezh2, neutrophils or precursors of DCs would be unable to disassemble adhesion structures, which would explain why these cells could not efficiently cross the blood-brain barrier in the EAE model or extravasate from the blood into inflamed tissues. In contrast to integrin-dependent extravasation^{2,3,43}, the migration of DCs from peripheral tissues to draining lymph nodes is a process that is largely dependent on actin polymerization and actomyosin contractions and not integrins³⁰. To determine whether Ezh2 aids the regulation of actomyosin-dependent migration of DCs *in vivo*, we applied the fluorescent dye FITC to the ears of control and $Ezh2^{f/f}$ *Cd11c*-Cre mice and analyzed draining lymph nodes for migrated dermal DCs 24 h later. In the $Ezh2^{f/f}$ *Cd11c*-Cre mice, Ezh2-deficient ($Ezh2^{\Delta/\Delta}$) DCs acquired the applied FITC dye and migrated to draining lymph nodes in a manner indistinguishable from that of control ($Ezh2^{f/f}$) cells (**Supplementary Fig. 8a**). Ezh2-deficient innate leukocytes also exhibited comparable cell size and actin structures to that of the control cells when they were attached to poly-L-lysine-coated surfaces through integrin-independent adhesion (**Supplementary Fig. 8b**). We suggest that the normal migration of Ezh2-deficient leukocytes in interstitial tissues was due not only to the utilization of integrin-independent migration processes but also to the lack of expression of interstitial collagen-binding integrins ($\alpha_2\beta_1$ and $\alpha_1\beta_1$)⁴⁴. Additionally, since integrin is not critical for the transmigration of cells of the hematopoietic lineage across the bone marrow sinusoidal barrier or their circulation through the blood stream in the steady state⁴⁵, we did not observe a reduction of circulating neutrophils or tissue-resident DCs (**Fig. 1**).

Ezh2-regulated integrin-dependent migration has also been demonstrated for epidermal Langerhans cells, whose migration across the basement membrane requires binding of Langerhans cell-expressed integrin α_6 to the integrin ligand laminin⁴⁶. In our experiments, Ezh2-deficient Langerhans cells failed to migrate through the basement membrane under both steady-state conditions and inflammatory conditions (**Supplementary Fig. 8c,d**). They also spread out extensively and formed enlarged focal adhesions on laminin coated slides (**Supplementary Fig. 8e,f**), similar to results obtained with BMDCs (**Fig. 3e,f** and **4a,b**). Moreover, the migratory defect of Ezh2-deficient (*Ezh2*^{Δ/Δ}) DCs was 'rescued' by lowering of the concentration of fibronectin coating on the plates (to 1.3 μg/ml), when the integrin-mediated adhesion was weakened (**Supplementary Fig. 8g,h**). In this case, the Ezh2-deficient (*Ezh2*^{Δ/Δ}) DCs did not spread extensively or form abnormal adhesion structures and therefore were able to overcome the restraining force caused by the lower rate of adhesion turnover and migrate in a fashion similar to that of the control (*Ezh2*^{ff}) cells (**Supplementary Fig. 8g-i**). Notably, the migration of both control DCs and Ezh2-deficient DCs under these conditions was inhibited by Y27632 (an inhibitor of ROCK, which is the downstream effector of Rho) and by blebbistatin (an inhibitor of myosin II) (**Supplementary Fig. 8h,i**), which indicated that the migration of mature DCs on two-dimensional surfaces required actomyosin contraction, similar to that observed under three-dimensional conditions³⁰. Collectively, our data revealed that Ezh2 was dispensable for actomyosin-dependent cell motility but was needed to regulate the integrin-dependent migration of leukocytes both *in vitro* and *in vivo* under steady-state or inflammatory conditions.

DISCUSSION

Here we have reported that Ezh2 interacted with Vav1 to promote the methylation of talin1, which altered the binding of talin1 to filamentous actin (F-actin) and exerted a major influence on the adhesion and migration of leukocytes both *in vitro* and *in vivo*. Because Vav1 is known to be required for the assembly of adhesion structures in various leukocytes³⁵, we hypothesized that the recruitment and activation of signaling molecules within adhesion structures leads to the dissociation of Vav1 from talin and thus enables dynamic reorganization of the adhesion structures. Even though the enlarged focal adhesions in Ezh2-deficient DCs were associated with enhanced formation of stress fibers, the Vav or small GTPase RhoA–ROCK signaling pathways were not regulated by Ezh2. The activation of PAK1, MLC2 and Vav was not affected by Ezh2 deficiency. Instead, our data suggested that the presence of Ezh2 in adhesion

structures directly contributed to the initiation of their disassembly through methylation of talin.

Since the Ezh2-interaction domain of Vav1 is highly conserved among members of the Vav family, we confirmed that the Ezh2-Vav-talin complexes that we observed in leukocytes also formed in non-hematopoietic cells (N.V., data not shown). The influence of Ezh2 on integrin-dependent adhesion and migration of cells is also probably not restricted to specific integrin molecules, because we observed substantial effects of Ezh2-mediated talin methylation on integrin function in several different cell types, including neutrophils (integrins β_2 and β_1), DCs (β_1) and epithelial cells (β_1)⁴⁴. Those data suggested that the mechanism described here for the regulation of adhesion and migration through the direct methylation of talin is probably conserved in other cell types. Our findings not only represent a substantial advance in understanding of the mechanisms that regulate leukocyte trafficking under steady state and inflammatory conditions but also provide a possible explanation for how Ezh2 can promote changes in cell adhesive properties during cellular transformation.

Although many identified lysine-methylation sites could be modified by ubiquitination, the Ezh2-mediated talin1 methylation site identified here (Lys2454) is predicted as a potential ubiquitination site only with a low confidence score (according to the CKSAAP_UbSite web server for the prediction of such sites). Therefore, it is unlikely that Ezh2-mediated methylation of talin1 competed with ubiquitination in this case. In addition, our data suggested that Ezh2-mediated methylation of Lys2454 occurred before the cleavage of talin and ubiquitin- and/or proteasome-regulated degradation of the talin head domain. We also identified additional Ezh2-mediated methylation site(s) within the talin dimerization domain. Since the dimerization domain is essential for actin binding⁴⁰, it is possible that Ezh2-mediated methylation of the dimerization domain interferes with dimerization and thereby, in addition to achieving methylation of Lys2454, further ensures less binding of actin and disassembly of adhesion structures. Further studies will now be needed to verify the methylation site (or sites) in the dimerization domain and determine whether such sites function synergistically in regulating adhesion dynamics.

Interestingly, post-translational modification of microtubules also affects focal adhesion dynamics. The acetylation of tubulin stabilizes focal adhesions, whereas deacetylation of tubulin mediated by the histone deacetylase HDAC6 promotes focal adhesion disassembly^{47,48}.

Details of the mechanism underlying this regulation remain unclear, but because the nuclear recombinant Ezh2 complex (EZH2, SUZ12, EED and PHF1) interacts with HDAC1 and HDAC2 through Eed⁴⁹, a component of the cytosolic Ezh2 complex¹⁹, it is feasible that the cytosolic Ezh2 complex recruits the major cytosolic deacetylase HDAC6 and regulates the turnover of adhesion structures. Thus, we are tempted to speculate that cytosolic Ezh2 complexes may be critical for the cross-talk between microtubules and the actin cytoskeleton.

It is likely that Ezh2 mediates the methylation of additional proteins to modulate other cytosolic signaling events. Ezh2 is involved in both the formation of circular dorsal ruffles in fibroblasts induced by the growth factor PDGF and actin polymerization triggered in T cells by the T cell antigen receptor (TCR)¹⁹, yet these processes are regulated by completely different mechanisms through different molecular targets. When the surface receptors were bypassed by chemical treatment with Y27632, blebbistatin or PMA plus ionophore, Ezh2-deficient cells responded normally. These results indicated that Ezh2 regulates the proximal signaling of various surface receptors. In addition, since Vav proteins are important for surface receptor proximal signaling, we suspect that they may have a crucial role in recruiting Ezh2 to targets other than talin in different cellular contexts.

Given our findings here, the defective TCR-mediated activation of Ezh2-deficient T cells might be a consequence of a dysregulated immunological synapse rather than an intrinsic flaw in actin polymerization¹⁹. Ezh2-deficient T cells have slightly elevated amounts of F-actin in the resting state and are still able to establish stable contacts with antigen-presenting cells. The efficient activation of T cells by antigen-presenting cells not only requires a stable interaction between these cell types but also depends on the dynamic reorganization of the TCR, co-stimulatory receptors, adhesion molecules and actin cytoskeleton⁵⁰. Therefore, Ezh2 may alter the strength of TCR triggering via the methylation of downstream signaling components, in addition to having effects on cell-cell adhesion structures. Together our data have demonstrated that interactions of Ezh2 with proteins of the Vav family and methylation of talin critically regulated the adhesion dynamics and integrin-dependent migration of leukocytes both *in vitro* and *in vivo* and thereby influenced the trafficking of cells between distinct tissue compartments and altering the outcome of inflammation and autoimmune disease.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Accession codes. GEO: microarray data, [GSE60146](#).

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

M.G. designed and conducted most of the experiments, interpreted data and was involved in manuscript preparation; N.V. and J.T.L. made major contributions to the manuscript revision; J.T.L. conducted experiments with DCs, neutrophils and Langerhans cells; N.V. and J.F.W. performed the *in vitro* methyltransferase assays, identified the Ezh2-Vav1 interaction mutant and did experiments with mammary epithelial cells; H.B. performed experiments with OT-II cells; L.Y.J.L. and L.G.N. performed intravital imaging of neutrophils; W.H.N. constructed talin1 expression vectors; T.G. and S.K.S. analyzed the mass spectrometry data; N.V. and M.K.L.W. produced the active and inactive Ezh2 (rPCR2) complexes; Y.H.Y. and S.G.S. performed the SPR analysis; P.C.E.S. and F.G. helped with the *in vivo* DC subset analysis; S.Y. was involved in setting up the *in vitro* DC cultures; K.C.C. provided microarray data; A.G. provided recombinant talin construct and advice on actin-binding assays; and I.S. designed and interpreted the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Figure 1 *Ezh2* is dispensable for the generation and distribution of DCs and neutrophils *in vivo*. **(a)** Flow cytometry of DCs isolated from the lungs, liver, spleen and inguinal lymph nodes (LN) of *Ezh2^{ff}* mice and *Ezh2^{ff}Cd11c-Cre* mice (*Ezh2^{Δ/Δ}*), gated on the basis of size, granularity, singularity and expression of the leukocyte marker CD45, then identified as CD11c⁺MHCII⁺ cells (top) and then subsorted on the basis of differential expression of CD103 and CD11b (below). Numbers above outlined areas (top) indicate percent CD11c⁺MHCII⁺ cells. Numbers in quadrants (bottom) indicate percent cells in each of the quadrants. **(b)** Flow cytometry of cells from the BM, spleen, ears and blood of poly(I)-poly(C) treated control (*Ezh2^{ff}*) and *Ezh2^{ff}Mx1-Cre* (*Ezh2^{Δ/Δ}*) mice, gated on the basis of size, granularity, singularity and CD45 expression. Numbers above outlined areas indicate percent CD11b⁺Ly6G⁺ cells (neutrophils). **(c)** Absolute number of DCs in **a** and number and frequency of neutrophils in **b**. Data are representative of more than three independent experiments (**a,b**) or three independent experiments (**c**; mean and s.d.).

Figure 2 Ezh2 is critical for the extravasation of innate leukocytes under inflammatory conditions. **(a)** Disease progression in 6-week-old female *Ezh2*^{f/f} mice ($n = 21$) and *Ezh2*^{f/f} *Cd11c*-Cre mice ($n = 19$) 0–25 d after induction of EAE, monitored daily (left), and frequency of DCs infiltrating into the inflamed central nervous system (CNS) at the peak of disease (day 21) in those mice (right). * $P = 0.0094$, ** $P = 0.0006$, ** $P = 0.001$, ** $P = 0.002$, * $P = 0.0061$ and * $P = 0.013$, left to right (left plot); ** $P = 0.002$ (right plot) (two-tailed student's t -test with equal variance). **(b)** Immunofluorescence microscopy of spinal cords from PBS perfused *Ezh2*^{f/f} mice (EAE score 3) and *Ezh2*^{f/f} *Cd11c*-Cre mice (EAE score 1.5) 21 d after induction of EAE, visualizing DCs with antibody to CD11c (anti-CD11c) (green), microvessels with anti-PECAM-1 (red) and nuclei with the DNA-binding dye DAPI (blue). Scale bars, 50 μ m. **(c)** CFSE profiles of donor OT-II T cells from *Ezh2*^{f/f} recipient mice (filled area) and *Ezh2*^{f/f} *Cd11c*-Cre recipient mice (solid line) given transfer of CFSE-labeled OT-II T cells (from mice deficient in the recombination-activating

gene 1), followed by immunized of the recipients with ovalbumin and LPS and analysis 3 d later. Dashed line, CFSE profiles of OT-II T cells in *Ezh2^{f/f}* control recipient mice treated with PBS instead of ovalbumin and LPS. **(d–f)** Ear inflammation in poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f} Mx1-Cre* mice 0, 2 or 4 h after induction by topical application of croton oil, with neutrophils identified in whole-mount ear staining by anti-Gr-1 **(d)** or anti-Ly6G, for flow cytometry **(e)**, or immunofluorescence staining **(f, left)**, and with the frequency of diapedesis after 3 h determined on the basis of z-stacks of confocal images **(f, right)**. Blood vessels **(d,f)** were visualized with anti-PECAM (green). Numbers adjacent to outlined areas **(e)** indicate percent CD11b⁺Ly6g⁺ cells. Scale bars, 100 μ m **(d)** or 50 μ m **(f)**. *** $P = 3.5 \times 10^{-7}$ (two-tailed student's *t*-test with equal variance). Data are from four independent experiments **(a)**; mean $\pm$ s.e.m. (left) or mean and s.d. (right)), are from one experiment representative of more than three independent experiments **(c)**; $n = 8$ mice per group) or are representative of three independent experiments **(b)** or more than four independent experiments **(d–f)**; mean and s.d. in **f**).

Figure 3 Ezh2 regulates the integrin-dependent adhesion and motility, but not the chemotaxis, of innate leukocytes. (a) Migration of mature BMDCs obtained from poly(I)-poly(C)-treated

Ezh2^{f/f} and *Ezh2^{f/f}Mx1-Cre* mice and made to adhere to fibronectin-coated slides, assessed in the presence (+CCL19) or absence (–CCL19) of CCL19 (upward arrows indicate position of CCL19 source), presented as trajectories (left) and summary of distance (top right) and directionality (bottom right). *** $P = 3.5 \times 10^{-5}$, 7.8×10^{-5} , 0.0001 and 0.0002 (right; top to bottom), and ** $P = 0.0036$ (two-tailed student's *t*-test with equal variance). **(b)** Migration of PMA-activated BM neutrophils from mice as in **a**. ** $P = 0.0043$ (left) and 0.0041 (right) (two-tailed student's *t*-test with equal variance). **(c)** Microscopy of DCs as in **a** without CCL19, outlined and tracked for 40 min. Scale bars, 45 μm . **(d)** Adherent serum-starved mature DCs from poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f}Mx1-Cre* mice, plated onto RGDS-coated plates for 5–60 min (horizontal axis), calculated on the basis of DAPI staining. Each symbol represents an individual mouse; small horizontal lines indicate the mean. * $P = 0.0062$, ** $P = 0.0014$ and *** $P = 1 \times 10^{-5}$ (two-tailed student's *t*-test with equal variance). **(e)** Cell area of serum-starved DCs from poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f}Mx1-Cre* mice, assessed on fibronectin-coated slides by time-lapse imagery. $P = 3.6 \times 10^{-124}$ (two-way analysis of variance (ANOVA) with replication). **(f)** Scanning electron microscopy of mature DCs from poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f}Mx1-Cre* mice, spreading on fibronectin-coated plates. Scale bars, 10 μm . **(g)** Size of immature and mature DCs in suspension, from poly(I)-poly(C)-treated *Ezh2^{f/f}* mice (blue lines) and *Ezh2^{f/f}Mx1-Cre* mice (red lines), assessed by flow cytometry of forward scatter (FSC). **(h)** Immunoblot analysis of talin1 (full-length (filled arrowhead) and cleavage product (rod domain; 190 kDa) (open arrowhead)), FAK phosphorylated at Tyr397 (p-FAK(Y397)), total FAK (arrow indicates cleavage product (100 kDa)) and tubulin (loading control) (left) and of FAK phosphorylated at Tyr576 and Tyr577 (p-FAK(Y576,Y577)), FAK phosphorylated at Tyr925 (p-FAK(Y925)), total FAK (arrow as at left) and tubulin in extracts of adherent mature DCs as in **d**. Data are from one experiment representative of three independent experiments (**a**, left, **b**, top, and **c**) or are pooled from three independent experiments (**a**, right, and **b**, bottom; mean $\pm$ s.e.m. of *Ezh2^{f/f}* DCs (47 without CCL19 and 23 with CCL19) or *Ezh2^{f/f}Mx1-Cre* DCs (51 without CCL19 and 27 with CCL19) in **a**, and $n = 114$ *Ezh2^{f/f}* neutrophils or $n = 100$ *Ezh2^{f/f}Mx1-Cre* neutrophils in **b**), are from three independent experiments with technical triplicates (**d**), are pooled from three independent experiments (**e**; mean $\pm$ s.e.m. of $n = 40$ cells per genotype), are representative of 3 experiments (**f**), more than 3 experiments (**g**) or 4 experiments (**h**).

Figure 4 Exaggerated cell adhesion in mature *Ezh2*-deficient DCs results from reduced turnover of adhesion structures. **(a)** Microscopy of mature BMDCs derived from poly(I)-poly(C)-treated *Ezh2*^{fl/fl} and *Ezh2*^{fl/fl} *Mx1*-Cre mice, plated for 45 min on fibronectin-coated slides and immunostained for F-actin or talin1 (green), talin1, vinculin or integrin (red) and DAPI (blue). Scale bars, 10 μ m. **(b)** Frequency of cells with large focal adhesions (Large FA), normal focal

adhesions or focal complexes (FA or FC) or no visible adhesion structures (No Ah) among cells as in **a**, and images of adhesions or lack thereof (below). Original magnification, $\times 60$. $*P = 0.0069$ and $***P = 0.0003$ (two-tailed student's *t*-test with equal variance). (**c,d**) Total internal refraction microscopy combined with time-lapse imaging of the interface between the cell and chamber surface, for of BMDCs obtained from poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f}Mx1-Cre* mice, transduced with retrovirus encoding GFP-paxillin and plated onto fibronectin-coated chambers; arrows indicate position of focal adhesions. Outlines in **c** (far left) indicate areas enlarged 4.5 times in **d**. Scale bars, 10 μm . (**e**) Microscopy of BMDCs obtained from poly(I)-poly(C)-treated *Ezh2^{f/f}* mice (top left) or obtained from poly(I)-poly(C)-treated *Ezh2^{f/f}Mx1-Cre* mice and untransduced (top middle) or transduced to express wild-type *Ezh2* (MIG-*Ezh2*), cytosolic *Ezh2* (MIG-*Ezh2*(Δ NLS)), enzymatically inactive *Ezh2* (*Ezh2*(H689A)) or *Ezh2* with diminished ability to interact with Vav1 (*Ezh2*(VavMT)), then plated onto fibronectin-coated slides and immunostained for paxillin (red) and DAPI (blue) (left), and formation of large focal adhesions in such cells (right). Scale bars (left), 5 μm . Average transduction efficiencies were comparable between constructs ($\sim 70\%$). $*P = 0.0065$, $**P = 0.0015$, 0.0033 and 0.0012 (left to right) and $***P = 0.0003$ (two-tailed student's *t*-test with equal variance). Data are representative of at least three independent experiments with 20–30 cells in each (**a**), three independent experiments with at least 100 cells per group in each (**b**), at least 60 cells per group in each (**e**) (**b,e**; mean and s.d.) or 3 experiments (**c,d**).

Figure 5 Ezh2 interactions with Vav1–tal1 promote talin1 methylation. **(a)** Reciprocal immunoprecipitation (IP) analysis of the interaction of Ezh2, talin1 and Vav1 in mature wild-type DCs. **(b)** Immunoprecipitation (IP) analysis of extracts of control and Ezh2-deficient BMDCs, derived from poly(I)-poly(C)-treated *Ezh2^{f/f}* and *Ezh2^{f/f} Mx1-Cre* mice, adherent on RGDS coated dish, analyzing the interaction of talin1 and Vav1. **(c)** Immunoprecipitation (IP) analysis of talin1 from mature wild-type BMDCs as in **b**, analyzing the associated Vav1 and Ezh2. **(d)** Immunoblot analysis of components of the active (rPRC2a) or inactive (rPRC2i) recombinant Ezh2 complex (EZH2, SUZ12, EED and PHF1) produced in Sf9 insect cells (above), and trimethylation of histone 3 lysine 27 (H3K27me3) in the histone octamer in an *in vitro* methyltransferase assay with the cold methyl-donor S-adenosyl methionine (bottom; control for activity of this complex). **(e,f)** *In vitro* methyltransferase assay with Ezh2 complexes (produced as described in **d**) with the radioactive methyl donor [³H]S-adenosyl methionine alone (**e,f**) or with talin1 immunoprecipitated from the murine DC2.4 cell line as a substrate (**f**), presented as a Coomassie gel (**e**, left) and fluorographs (**e**, right, and **f**); the low-molecular-mass

bands (right two lanes in **f**) are nonspecific and probably resulting from other methyltransferase(s) that precipitated together with talin1. (**g**) Immunoblot analysis of methylated talin1 (Me-talin1), total talin1, and GFP-tagged Ezh2 in HEK293T cells left untransfected (Un) or transfected to express wild-type Ezh2 (WT) or the 'enzymatically hyperactive' Ezh2 mutants A677G or Y641F, assessed immunoprecipitation of endogenous talin1, and probed with antibody to total methylated lysine. Close and open triangles indicate the position of full length talin1 and talin1 rod domain, respectively. (**h**) Immunoblot analysis of talin1 methylation (as in **g**) in HEK293T cells in which Vav2 expression was knocked down via short hairpin RNA (Vav2-KD), and in wild-type cells (WT). Data are representative of more than three independent experiments (**a-h**).

Figure 6 Ezh2-mediated methylation of talin1 enhances the cleavage of talin1. (**a**) *In vitro* methyltransferase assay of biotinylated peptides derived from various regions of talin1 (amino acid ranges, above lanes) conjugated to streptavidin agarose beads (as in **Fig. 6e**), presented as a flurograph (top) and the corresponding Coomassie gel (bottom). Arrowhead indicates self-

methylation of Ezh2 in the active (PRC2a) complex; arrows indicate positions of methylated peptides of expected size; *, peptide resulting from intermolecular disulfide bonding between cysteine residues (extra band eliminated by treatment with iodoacetamide before mass spectrometry). **(b)** The Ezh2-mediated talin1 methylation site, identified by mass spectrometry and presented as precursor ion spectra of unmodified peptides and peptides modified by trimethylation at Lys15 (K15Me3) (42.05-Da shift) from their respective retention fractions in 5+ charge state (detailed methylation-site assignment, **Supplementary Fig. 6** and **Supplementary Tables 1** and **2**). **(c)** Immunoblot analysis of full-length talin1 (Talin1-F) and the talin1 rod domain (Talin1-R), and head domain (Talin1-H), as well as Ezh2 and GAPDH (loading control), in MCF10a cells left untransduced (far left) or transduced to express short hairpin RNA targeting endogenous Ezh2 alone (Ezh2-KD) or together with vector for the expression of full-length Ezh2 or various EZH2 variants (as in **Fig. 4e**). **(d)** Immunoblot analysis of full-length talin1, the talin1 head domain and tubulin in HEK293T cells transduced to transiently and exogenously express fusions of talin1 (wild-type or the K2454Q or K2454F mutant) with GFP at the amino terminus, assessed with anti-GFP. **(e)** Immunoblot analysis of full-length talin1, methylated talin1, talin1 trimethylated at Lys2454 (K2454me3) and GFP-tagged Ezh2 in HEK293T cells transduced to transiently express empty vector (EV), cytosolic, enzymatically inactive Ezh2 (Ezh2(Δ NLS,H689A)), cytosolic Ezh2 (Ezh2(Δ NLS)) or a 'hyperactive' Ezh2 mutant (Ezh2(Y641F)) in the presence of the calpain inhibitor E-64d, after immunoprecipitation of endogenous talin1, probed with antibody to total methylated lysine or antibody specific for trimethylation at Lys2454 (**Supplementary Fig. 7e–g**). Data are representative of three independent experiments **(a)** or more than three independent experiments **(b–e)**.

Figure 7 Ezh2 regulates adhesion dynamics through methylation of talin1. **(a)** Electrostatic profiling of the talin1 carboxy-terminal actin-binding domain and the position of Lys2454 (yellow shading). **(b)** Microscopy (top) of H16N2 cells in which endogenous Ezh2 was knocked down, transduced with retrovirus for the expression of full-length Ezh2 or various EZH2 variants (as in **Fig. 4e**), showing focal adhesion structures (arrows) visualized by vinculin staining (red) and nuclei stained with DAPI (blue); bottom left, frequency of cells (as above) with large focal adhesions (filled black bars), normal focal adhesions (filled gray bars) or no focal adhesions (open bars). Scale bars (top), 20 μ m. **(c)** Microscopy of MCF10a cells transduced to express

retrovirus encoding GFP-tagged cytosolic Ezh2 (Ezh2(Δ NLS)-GFP) or cytosolic, enzymatically inactive Ezh2 (Ezh2(Δ NLS-H689A)-GFP) and mCherry-paxillin; outlines indicate areas 4 times enlarged below. Scale bars, 20 μ m. **(d)** Quantitative analysis of the binding of talin1 mutants (key) to various concentrations of F-actin (horizontal axis), determined by actin co-sedimentation. $**P = 0.0042$ and $***P = 4.7 \times 10^{-9}$ (two-way ANOVA with replication,). **(e)** Adhesion structures and F-actin in H16N2 cells transduced with virus expressing wild-type GFP-tagged wild-type talin1 or the K2454A, K2454Q or K2454F talin1 mutants, visualized by immunostaining with anti-GFP (green) and phalloidin (red), and by staining of nuclei with DAPI (blue). Scale bars, 10 μ m. **(f)** Frequency of H16N2 cells expressing talin1 variants (key) without (w/o) any visible adhesion structures. $***P = 0.0002$ (one-way ANOVA). **(g)** Quantification and size of nascent adhesions (NAs), focal complexes, focal adhesions and fibrillar adhesions (FB) in H16N2 cells transduced as in **e** (measured as defined in **Supplementary Fig. 4e**). (one-way ANOVA, $**P = 0.001$, $***P = 10^{-5}$). Data are representative of three independent experiments with 50–60 cells per group (**b**), 3 experiments (**c**, **e**) or are from five independent experiments (**d**; mean $\pm$ s.d.) or three independent experiments (**f**, **g**; mean $\pm$ s.d. of $n > 100$ cells per genotype).

Figure 8 Trimethyl lysine mimicking mutant talin1 rescues excessive cell spreading and defective cell migration phenotypes of Ezh2-deficient DCs. **(a)** Area of control and Ezh2-

deficient BMDCs (derived from poly(I)-poly(C) pre-treated *Ezh2^{f/f}* and *Ezh2^{f/f}* Mx1-Cre mice) left untransduced (Un) or transduced to express GFP-tagged talin1 variants (key), allowed to adhere for 2 h to slides coated with various concentrations of VCAM-1 (5, 10 and 20 $\mu\text{g/ml}$; above plots), visualized by GFP staining. Black asterisks (left to right; within the group), $***P = 3.9 \times 10^{-27}$, 1.6×10^{-10} , 1.2×10^{-19} , 2.5×10^{-17} , 5.2×10^{-7} and 1.6×10^{-16} (one-way ANOVA); red asterisks (left to right; pairs), $***P = 9.9 \times 10^{-10}$, 3.1×10^{-8} , 2.8×10^{-7} , 4.4×10^{-23} and 6.9×10^{-8} , and $*P = 0.024$ or 0.031 (two-tailed student's *t*-test with equal variance). **(b)** Migration of *Ezh2^{f/f}* and *Ezh2*-deficient DCs left untransduced or transduced to express talin1 variants (horizontal axes), assessed after adherence for 1 h to slides coated with various concentrations of VCAM-1 (as in **a**), with time-lapse images obtained every 5 min for an additional 2 h (assessed as described in **Fig. 3a**). $***P = 1.3 \times 10^{-7}$, 2.1×10^{-21} , 5.3×10^{-5} , 10^{-12} , 7.6×10^{-6} and 1.8×10^{-5} , or $*P = 0.0064$, left to right (two-tailed student's *t*-test with equal variance). Data were pooled from two (5 or 20 $\mu\text{g/ml}$) or four (10 $\mu\text{g/ml}$) independent experiments with 30 cells **(a)** or 100 cells **(b)** per genotype in each (mean $\pm$ s.e.m.).

ONLINE METHODS

Mice. Deletion of the *Ezh2* locus in *Ezh2^{f/f}* Mx1-Cre mice was achieved as described²³. *Cd11c*-Cre mice, originally obtained from The Jackson Laboratory (USA), were crossed with *Ezh2^{f/f}* mice to generate offspring with *Ezh2*-deficient DCs *in vivo*. All mice were bred and maintained under specific pathogen-free conditions at the animal facility of the School of Biological Sciences, Nanyang Technological University. All mouse protocols were conducted in accordance with the guidelines of the Nanyang Technological University Institutional Animal Care and Use Committee.

EAE induction and assignment of scores. A peptide of myelin oligodendrocyte glycoprotein (MOG) amino acids 35–55 was synthesized and purified to a purity of >99% by high-performance liquid chromatography at the School of Biological Sciences, Nanyang Technological University core facility. The lyophilized MOG peptide was then dissolved in sterile PBS at 2mg/ml. *Mycobacterium tuberculosis* strain H37 Ra (Difco) was dissolved in incomplete Freund's adjuvant (Sigma-Aldrich) at a concentration of 11 mg/ml. MOG peptide solution and complete Freund's adjuvant solution were then mixed at a ratio of 1:1 by repeated forcing of the mixture through an 18-gauge needle to generate a homogenous suspension before subcutaneous injection of a 100- μl volume into 6-week-old female *Ezh2^{f/f}* and *Ezh2^{f/f}* *Cd11c*-Cre mice. *Bordetella pertussis* toxin (10 $\mu\text{g/ml}$; Roche) in pyrogen-free H₂O was injected intraperitoneally immediately after the peptide injection and then again 8 h later. The whole process was repeated 7 d after the first injection. Mice were monitored and assigned scores daily for signs of EAE according to standard protocols over a total period of 25 d, as follows: 0, no EAE; 1, limp tail; 2, partial hind and/or front leg paralysis; 3, total hind leg paralysis; 4, total hind leg and partial front leg paralysis; 5, moribund, death.

CFSE labeling and *in vivo* T cell stimulation. OT-II T cells deficient in recombination-activating gene 1 were purified and labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) according to standard protocols. The labeled T cells were transferred intravenously into control (*Ezh2^{f/f}*) and *Ezh2^{f/f}* *Cd11c*-Cre mice. Recipient mice were immunized with ovalbumin protein (50 $\mu\text{g/mouse}$) and LPS (25 $\mu\text{g/mouse}$) 24 h after cell transfer. CFSE profiles were analyzed 3 d later by flow cytometry.

Ear inflammation and whole-mount ear staining. Cutaneous inflammation was induced in mice by topical application of croton oil (1% in acetone) with a micropipette (20 $\mu\text{l/ear}$) to the outer surface of the right ear. Mice were killed at the appropriate time points. Split ear halves were fixed in acetone for 20 min at -20°C and were stained overnight at 4°C with anti-CD31 (MEC 13.3; BD Biosciences) and anti-Ly-6G (1A8; Biolegend). Ear halves were mounted with mounting medium (Ibidi) containing DAPI (4,6-diamidino-2-phenylindole; Invitrogen). *z*-stacks of confocal images were acquired.

Isolation and culture of BMDCs. BM cells were harvested from *Ezh2^{f/f}* mice and *Ezh2^{f/f}* Mx1-Cre mice by standard protocols before being cultured in complete RPMI-1640 medium (Gibco-BRL) supplemented with 20 ng/ml of the cytokine GM-CSF and 10ng/ml IL-4 (Prospec Tany). On day 2 of culture, the cells were gently washed and the medium replaced with fresh complete medium supplemented with 10 ng/ml GM-CSF. Cells were cultured for 6 or 7 d and medium was added or refreshed as necessary. For the generation of mature DCs, the immature cells were stimulated overnight in the presence of 100 ng/ml LPS (Sigma-Aldrich). In some experiments, mature DCs were starved for 6 h in serum-free medium before analysis.

Organ collection and preparation. *Ezh2^{fl/fl}* and *Ezh2^{fl/fl} Cd11c-Cre* mice were killed for the collection of spleen, lungs, liver and inguinal lymph nodes. The lymph nodes were disrupted with forceps, and all other tissues were cut into small pieces before incubation in 0.1 mg/ml collagenase type 4 (Sigma-Aldrich) in Hank's buffered salt solution. Tissue fragments were then homogenized and filtered through a cell strainer (BD Falcon). The liver cells were further purified by centrifugation over Percoll gradients (GE Healthcare). Spinal cords were isolated by perfusion of the mice with PBS, followed by flushing of the spinal cord from spine. Spinal cord tissues were floated in collagenase type IV, then were homogenized and filtered and then purified with a Percoll gradient (GE Healthcare).

Cell motility and chemotaxis assay. Mature DCs or neutrophils were allowed to adhere to culture slides (BD Biosciences) coated with fibronectin (10 µg/ml; Sigma-Aldrich), VCAM-1 (10 µg/ml) or ICAM-1 (12.5 µg/ml; Sino Biological). Non-adherent cells were removed by gentle washing, and the adherent DCs were supplemented with fresh complete medium with or without 100 ng/ml CCL19 (DENOVO). Images of live cells were recorded at a time-lapse interval of 10 min over a period of 5 h. Individual cell trajectories were analyzed with Metamorph object-tracking software (Molecular Devices). Tracking was restricted to cells that remained visible within the observation field for more than half the total duration of the experiment. For chemotaxis assays, mature DCs were plated onto fibronectin-coated µ-slide chemotaxis chambers (Ibidi). Gradients were established by the addition of 100 ng/ml CCL19 (or medium only; control) to one end of each chamber. Culture slides were maintained at 37 °C in 5% CO₂ on a microscope stage. Cells were visualized at a time-lapse interval of 10 min for a total time of 2 h. Visualization and image acquisition were performed as described above. Cell-migration tracks, distances traveled and directionality were analyzed with ImageJ software (National Institute of Health) with a manual tracking plug-in and the chemotaxis-migration tool (Ibidi).

Immunofluorescence staining. Immunofluorescence staining was performed according to standard protocols, with anti-talin (8D4; Sigma), anti-vinculin (hVIN-1; Sigma) and anti-paxillin (5h11; Millipore). F-actin was stained with fluorescein isothiocyanate- or rhodamine red-conjugated phalloidin (Invitrogen). Slides were mounted with mounting medium (Ibidi) containing DAPI (Invitrogen).

Microscopy. Confocal images were acquired and analyzed with a LSM510 or LSM710 confocal system based on an Axiovert 200M inverted microscope (Carl Zeiss) with Axiovision 4.6 software. Confocal images were captured at a magnification of ×63. A system based on a TE-2000 inverted microscope (both from Nikon) was used for total internal reflection microscopy, and images were captured with an iXon EMCCD camera (Andor Technology) at a magnification of ×60 for analysis with NIS-Element software (Nikon). Live cells were imaged with an Axiovert inverted microscope (Carl Zeiss) at a magnification of ×10 or ×63. Culture slides were maintained at 37 °C and 5% CO₂ on the microscope stage. Images were acquired with a Coolsnap charge-coupled device camera (Photometric) and the MetaMorph Imaging System 4.0 (Universal Imaging). Time-lapse images were reconstructed as image stacks (MetaMorph). Bright-field images of crystal violet-stained cells were acquired with a TS100 inverted microscope and a DS color charge-coupled device camera (both from Nikon). Images were acquired and analyzed with NIS elements software (Nikon).

Retroviral transduction of BMDCs. BMDCs were transduced with retrovirus based on the mouse stem cell virus (MSCV)-internal ribosomal entry site (IRES)-GFP (MIG) vector encoding *Ezh2*, cytosolic *Ezh2*, enzymatically inactive *Ezh2* or the *Ezh2*-Vav interaction mutant. Cells were transduced on day 3 and day 4 of culture by spin infection in the presence of 8 µg/ml polybrene. LPS-matured BMDCs were collected on day 6 or day 7 and then were allowed to adhere to fibronectin-coated µ-slide chambers (Ibidi) before the addition of reagents to stain talin, paxillin or vinculin. Cells were analyzed by laser-scanning confocal microscopy as described above. The transduction efficiency was routinely assessed by flow cytometry of GFP expression (mean 70–80% of cells transduced). Approximately 60 cells were assigned scores for statistical analysis (Student's *t*-test). For time-lapse imaging of focal adhesion turnover, mature DCs were transduced with retrovirus encoding GFP-paxillin. Transduced cells were seeded onto fibronectin-coated µ-slide chambers (Ibidi) and were analyzed by total internal reflection microscopy. For assessment of adhesion complexes in mature DCs, images were captured with a time interval of 5 min over a total period of 70 min; for assessment of podosome turnover in immature DCs, we used a time interval of 30 s over a total period of 10 min. All retroviral constructs were constructed with the MIG vector, which contains an IRES-GFP reporter. The transduction efficiency was routinely monitored by flow cytometry.

Cell adhesion and spreading assay. Mature DCs adherent to plates coated with RGDS (10 µg/ml; Merck) were fixed and stained with 0.1% crystal violet in 10% ethanol. Images were captured by bright-field illumination at a magnification of ×20. Cells were stained with DAPI to allow quantification of adherent cells without cell area bias, and nuclei in three randomly selected fields were counted manually. For the spreading assays, cells were seeded onto fibronectin-coated µ-chambers (Ibidi) and were maintained at 37 °C and 5% CO₂ on the microscope stage. Immediately after seeding, cells were visualized using an inverted microscope (Carl Zeiss Inc) with a 63× DIC objective (Carl Zeiss Inc). Images were acquired as described above at a time-lapse interval of 5 min for a total duration of 205 min.

Electron microscopy. *Ezh2^{fl/fl}* and *Ezh2^{Δ/Δ}* mature DCs were added to six-well plates and were allowed to adhere for 1–2 h at 37 °C. The adherent cells were fixed with 2% glutaraldehyde, post-fixed with 0.1% osmium tetroxide, dehydrated in ethanol, critical point-dried, and then sputter-coated with gold and palladium. Images were obtained with a LEO 1550 scanning electron microscope (Carl Zeiss).

In vitro methyltransferase assay. The baculovirus-derived recombinant *Ezh2* complex (rPRC2) complex consisting of *Ezh2*, Suz12, EED and PHF1 was produced in Sf9 insect cells and then purified. Methylation reactions were conducted with the enzymatically active complex or the enzymatically inactive complex (with substitution of alanine for histidine at position 698 (H689A) in *Ezh2*). Talin was immunoprecipitated from DC2.4 cells with anti-talin (Sigma-Aldrich) and was incubated with the active complex or the inactive complex in methylation buffer (50 mM Tris, pH 8.0, 1mM PMSF and 0.5 mM DTT), containing 7.6 µM (15 µCi) *S*-adenosyl-L-[methyl-³H]methionine ([³H]SAM). The reaction was carried out, with gentle shaking at 280 r.p.m., for 1 h at 30 °C. The reaction was stopped by the addition of 2× SDS gel loading buffer followed by separation of PRC2 complexes and substrates by SDS-PAGE. Gels were stained with Coomassie Brilliant blue, and the ³H signal was enhanced with EN3HANCE according to the manufacturer's instructions (PerkinElmer). Gels were exposed to X-ray film (hypersensitive films; Amersham) for 7–10 d at –80 °C. The film was then developed in a Kodak processor.

Fluorescein isothiocyanate painting. Fluorescein isothiocyanate (Sigma) was suspended in a solution of acetone and dibutyl phthalate (at a ratio of 1:1) to achieve a final concentration of 0.5%. One ear of each *Ezh2^{fl/fl}* and *Ezh2^{fl/fl}Cd11c-Cre* mouse was painted with 20 μ l fluorescein isothiocyanate. After 24 h, the mice were killed and the draining lymph nodes were collected. Single-cell suspensions were prepared from the lymph nodes by digestion with Liberase blendzyme (Roche). Cells were then stained for analysis by flow cytometry.

Actin co-sedimentation assays. Purified rabbit skeletal actin (Cytoskeleton) was polymerized for 60 min at room temperature in 10 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl₂ and 1 mM ATP. Recombinant histidine-tagged Talin1(2300–2541) was purified with nickel beads, followed by digestion with TEV protease. Wild-type talin or various talin mutants (4 μ M) were incubated for 60 min at room temperature with F-actin (ranging from 0 μ M to 10 μ M), followed by centrifugation at 150,000g for 30min at 24 °C. Supernatants and pellets were separated by 12% SDS-PAGE, then the gels were stained with Coomassie blue and scanned. Protein abundance in the pellets was determined with Image J software (NIH) and was normalized to that of talin loading controls.

Surface plasmon resonance measurement. Surface plasmon resonance was measured on a Biacore 3000 (GE Healthcare) at 25 °C. Histidine-tagged talin1(2300–2541)-Lys2454 and its mutants (K2454Q or K2454F) were separately pre-incubated for 60 min with F-actin (2.5 μ M) in the presence of phalloidin (2.5 μ M) to allow complex formation. These complexes were then captured onto sensorchips (GE Healthcare) coated with pre-immobilised antibody to penta-histidine (34660, Qiagen) at a flow rate of 5 μ l/min until total protein complexes were captured by antibody (the refractive unit reaches 400RU $\pm$ 20 among the 3 different complexes). Complexes formed with histidine-tagged talin (wild-type or mutant) at a concentration of 4, 20 or 40 μ M were captured onto different flow cells separately to maintain identical dissociation conditions with a running buffer containing 20 mM Tris and 90 mM NaCl, pH 8.0. Dissociation was monitored for at least 16 h. Histidine-tagged talin (wild-type or mutant) pre-incubated with actin monomer under the same condition were run as controls. The complex-dissociation response obtained was corrected through the analysis of controls, and the rate of dissociation was fit to first-order kinetics.

Microarray and data analysis. BMDCs (untreated and stimulated with LPS) were lysed with Trizol (Life Technologies, Invitrogen), and total RNA was prepared with an RNeasy kit (Qiagen). Biotinylated cRNA was prepared from 50 ng of total RNA with a TargetAmp Nano-g Biotin-RNA labeling kit for the Illumina System (Epicentre). Labeled cRNA was hybridized to Illumina Mouse Ref-8v2.0 Beadchips, and these arrays were then washed and stained according to the Illumina wash protocol. The arrays were then scanned with a BeadArray Scanner 500 GX, and the images were analyzed with the GenomeStudio Gene Expression integrated platform. The intensity values of the arrays were extracted without subtraction of background with BeadStudio software. Intensities with a detection *P* value of >0.05 were replaced by the lowest positive intensity with detection *P* value equal to an interquartile range filter of 0.40. Differentially expressed genes were identified in this subset with limma, with a Benjamini-Hochberg false-discovery rate of 0.05 and an absolute difference in expression of at least twofold. All data analysis was enabled with the Pipeline Pilot informatics platform.

Supplementary Figure 1

Efficient deletion of *Ezh2* gene and Ezh2 protein in DCs and neutrophils.

(a) Deletion efficiency of *Ezh2* gene in *ex vivo* isolated DCs. CD11c^{high} and CD11c^{low} cells were sorted from skin, lymph nodes and spleen of the *Ezh2^{flΔ}* and *Ezh2^{flfl}* *Cd11c*-Cre mice. RNA was isolated and used for cDNA synthesis followed by specific PCR (full-length *Ezh2* transcript: 543 bp, truncated *Ezh2* transcript: 246bp). HPRT was used as a loading control. (b) Cell numbers of spinal cord infiltrating leukocytes (top) and percentages of DCs associated with blood vessels (bottom) at the peak of EAE disease (day 21) in *Ezh2^{flfl}* control and *Ezh2^{flfl}* *Cd11c*-Cre mice are shown. Error bars indicate mean $\pm$ s.d. of 3 independent experiments. **P*=0.01. (c) Deletion efficiency of *Ezh2* gene in neutrophils. Gr-1⁺ granulocytes including neutrophils (Nph) were isolated from bone marrow (BM) and peripheral blood (PB) of poly(I)-poly(C) treated *Ezh2^{flfl}* and *Ezh2^{flfl}* *Mx1*-Cre mice. RNAs from purified cells were processed as described above. (d) Generation of Ezh2-deficient BMDCs *in vitro*. Bone marrow cells from *Ezh2^{flfl}* and *Ezh2^{flfl}* *Mx1*-Cre mice pre-treated with poly(I)-poly(C) were isolated and cultured in the presence of GM-CSF and IL-4. The phenotypes of the CD11c⁺ cell populations were analyzed by FACS on day 3, 5 and 6, with or without the final 20 h LPS treatment. Filled gray area indicates the fluorescence content of the isotype control. (e) Deletion efficiency of the floxed *Ezh2* locus in BMDCs. RNAs from *Ezh2^{flfl}* and *Ezh2^{Δ/Δ}* BM and BMDC were isolated and the deletion efficiency was determined as described in a. (f) Depletion of Ezh2 protein in Ezh2-deficient BMDCs. Expression of Ezh2, Suz12 and Vav1 in nucleus and cytosol was analyzed in BMDCs isolated from culture at day 6 (lane 1, 4) and day 8 with (lane 3, 6) or without LPS (lane 2, 5) stimulated maturation. Expression of lamin B and tubulin was used to control for the purity of the nuclear and cytosolic fractions, respectively.

Supplementary Figure 2

Ezh2-deficient mature DCs display comparable H3K27me3 content and gene-expression profiles.

(a) The content of H3K27me3 of cultured bone marrow derived mature DCs (day 8) from poly(I)-poly(C) treated $Ezh2^{fl/fl}$ (solid line) and $Ezh2^{fl/fl}$ Mx1-Cre ($Ezh2^{\Delta/\Delta}$) (dashed line) mice were analyzed by intracellular staining (H3K27me3) and flow cytometry. Isotype control is indicated by filled area. Gated mature DCs (CD11c⁺Class II^{hi}) are shown in these histograms. (b-d) Gene expression patterns in control and Ezh2-deficient BMDCs. RNA isolated from control ($Ezh2^{fl/fl}$) and Ezh2-deficient ($Ezh2^{\Delta/\Delta}$) DCs were processed according to manufacturer's instruction. Samples were hybridized to Illumina WG6v2 mouse arrays. Raw intensities were extracted using Bead Studio, and quantile normalized. Differential gene expressions were determined with limma, using BH multiple test correction and an FDR cutoff of ≤ 0.05 . All data processing and analysis were performed using Accelrys Pipeline Pilot (www.accelrys.com). Biological triplicates were done for each group. (b) Venn diagram showing the overlap in differentially expressed genes (DEGs) between control dendritic cells with versus without LPS stimulation, and Ezh2-deficient DCs with versus without LPS stimulation. Control DC specific DEGs were 29 and Ezh2-deficient DC specific DEGs were 28. In total, 98% of the DEGs (2643) were in common between control and Ezh2-deficient DCs. (c) Scatter plot of $\log^2$, normalized intensities from the array of a representative experiment of bone marrow derived control versus Ezh2-deficient immature DCs (iDCs). A Pearson's r correlation of 0.9947 was obtained. (d) Scatter plot of $\log^2$, normalized intensities from the array of a representative sample of control versus Ezh2-deficient LPS-stimulated mature DCs (mDCs). A Pearson's r correlation of 0.9932 was obtained.

Supplementary Figure 3

Defective migration of Ezh2-deficient leukocytes is not caused by impaired responsiveness to chemoattractant.

(a) Migration of neutrophils on ICAM-1-coated slides in the absence (black bar) or presence (white bar) of fMLP. Control and Ezh2-deficient neutrophils isolated from the bone marrow of poly(I)-poly(C) treated *Ezh2^{fl/fl}* and *Ezh2^{fl/fl}* Mx-Cre mice were purified using murine neutrophil isolation kit (Miltenyi). Purified neutrophils were seeded to ICAM-1-coated chamber slides and stimulated with fMLP (10^{-5} M) for 10 minutes in 37°C. Non-adherent cells were removed, adherent cells were used in parallel for time-lapse imaging at 30-sec interval for 30 min. Phase contrast images were captured using Zeiss inverted microscope at 10x magnification with temperature and CO₂ controls. Only cells that were not clustering and could be tracked for the whole time duration were taken into account. Cell tracking was done using Image J software, with Manual Tracking, and Chemotaxis Plugin. About 100 cells from 3 random fields were tracked in each experiment. Statistical analysis of distance was compiled using a "Chemotaxis Plug-in tool" from ImageJ. Error bars represent mean $\pm$ s.d., *** $P=1.1 \times 10^{-19}$. **(b)** Control (solid line filled square) and Ezh2-deficient (dashed line open circle) bone marrow neutrophils were rested at room temperature for 1 hour before pre-warming them at 37°C for 10 min and stimulated in solution with fMLP for indicated time points. Actin contents were measured by phalloidin staining followed by FACS analysis. **(c)** Activation of integrin downstream signaling molecules in control and Ezh2-deficient neutrophils. Neutrophils were stimulated as described in **b**. Equal amount of lysate was resolved with SDS-PAGE, and probed with indicated antibodies. **(d)** The surface expression of CCR7 on control (blue line) and Ezh2-deficient CD11c⁺MHCII^{hi} mature DCs (red line) derived from the bone marrow of poly(I)-poly(C) treated *Ezh2^{fl/fl}* and *Ezh2^{fl/fl}* Mx-Cre mice were analyzed by flow cytometry. Isotype control is indicated by filled area.

Supplementary Figure 4

Rapid spreading and activation of Ezh2-deficient innate leukocytes.

(a) Control (*Ezh2^{fl/fl}*) and Ezh2-deficient (*Ezh2^{Δ/Δ}*) mature DCs derived from poly(I)-poly(C) treated *Ezh2^{fl/fl}* and *Ezh2^{fl/fl}* Mx1-Cre mice were serum-starved and plated onto RGDS-coated plates for the indicated periods of time. Adherent cells were fixed and stained using crystal violet and DAPI. Data shown are representative of 3 independent experiments with triplicates. The corresponding statistical analysis is shown in **Fig. 3d**. (b) The surface expression of integrins $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_x\beta_2$, and $\alpha_M\beta_2$ in control (blue line) and Ezh2-deficient DCs (red line) were analyzed by flow cytometry. Isotype control is indicated by filled area. Gated mature DCs (CD11c⁺MHCII^{hi}) are shown in these histograms. (c) Control and Ezh2-deficient mature BMDCs as in **a** were plated onto RGDS coated plate for indicated time points. Equal amount of lysate was resolved by the standard SDS-PAGE, and probed with indicated antibodies. "L" indicates cells adherent to poly-L-lysine coated dishes for 45 min. (d) Control (*Ezh2^{fl/fl}*) and Ezh2-deficient (*Ezh2^{Δ/Δ}*) neutrophils isolated from the BM of poly(I)-poly(C) treated *Ezh2^{fl/fl}* and *Ezh2^{Δ/Δ}* Mx1-Cre mice were rested as described in **Supplementary Fig. 3b** and plated onto ICAM-1 coated dishes for indicated time points. Activation of indicated molecules was determined by specific antibodies. (e) Control and Ezh2-deficient mature DC as in **a** adherent to VCAM-1-coated slide were stained for talin1 (red) and statistical analysis of various adhesion structures (bottom) in control (filled bars) and Ezh2-deficient DCs (open bars) were performed. Nascent adhesions (NAs), focal complexes (FCs), focal adhesions (FAs) and fibrillar adhesions (FBs) were measured by setting the size limits of the measured particles from 0.05-200 μm^2 . The classifications are as follows: NA <0.25 μm^2 , FC <0.5 μm^2 , FA <1.5 μm^2 , FB > 5 μm^2 . Error bars represent Mean $\pm$ s.e.m. of cells pooled from 3 independent experiments. (Two tailed student's t test, * $p=0.041$ (left), 0.008, *** $p=0.0002$ (left), 5×10^{-8}). (f) The expression of Paxillin, p-Paxillin (Y118) or overall phosphorylated protein (Py99) in control and Ezh2-deficient DCs on fibronectin coated slides was determined by immunofluorescence staining and the images were acquired either with confocal or TIRF microscopy. (g) Immunofluorescence staining of indicated proteins in control and Ezh2-deficient neutrophils on ICAM-1 coated slides. Paxillin (red), p-Paxillin (Y118) (green), DAPI (blue). Data shown in this figure are either representative from 1 of 3 (**a**, **b**, **e** (top), **f**) or 2 (**g**, **d** with 3 mice per group) independent experiments or summary (**e**, bottom) of data acquired from 3 mice per group. Scale bars in **e**, **f**, **g** represent 10 μm .

Supplementary Figure 5

Substitutions of Lys241 and Lys245 in Ezh2 disrupt interactions of Ezh2 with Vav1.

(a) Ezh2–Vav1 interaction domains, Ezh2 (Ezh2₂₀₁₋₂₅₂) and Vav1 (Vav1₁₋₁₇₂) with C-terminal His-tag, respectively, were purified from *E. coli*. Mutations of crucial residues (through computational prediction) for this interaction were introduced into Ezh2₂₀₁₋₂₅₂ and the interactions of these mutated recombinant proteins with Vav1 were tested in His pull down experiments. Ezh2₂₀₁₋₂₅₂K241/245A mutant was referred to as Ezh2VavMT in the main text. The enzymatic activity of Ezh2 is not affected by this mutation (Venkatesan et al., unpublished data). (b) Methylation of endogenous talin1 from cells expressing wild-type Ezh2 (Ezh2), hyperactive Ezh2 (Ezh2Y641F) or Vav interaction mutant Ezh2 (Ezh2VavMT) were determined as described in Fig. 5g. Data shown in this figure are representative from one of 3 independent experiments. (c) The effects of Vav interaction mutant Ezh2 on its co-localization with focal adhesions were determined by transfecting indicated Ezh2 and mCherry-paxillin constructs into MCF10a cells and the fluorescence signals were measured by confocal microscopy. White boxes indicate the regions enlarged in the following panels (box 1 middle panel, box 2 lower panel). Scale bars, 20 μm.

Supplementary Figure 6

Ezh2 mediates trimethylation of talin1 at Lys2454.

ETD-MS/MS spectra were recorded by Orbitrap Elite in the supplemental activation ETD mode. MS/MS spectra of the unmodified peptide (Red) and the K15Me3 modified peptides (Blue) were displayed in the stack plot with Y-offset of 10. The supplemental activation ETD fragmentation of peptide produced mainly *c* and *z*[•] ions, with some *b* and *y* ions. The ions of the small fragments of the two peptides that do not contain the K15Me3 (talin1-K2454) have the same masses (*m/z*) and were labeled in black. The major *c* and *z*[•] ions of larger fragments that contain the K15 (Red) from the unmodified peptide or K15Me3 (Blue) from the modified peptide were labeled. The spectra showed a systematic 42 Da shift in the fragment containing the K15Me3 modification. The K15 modification site is sandwiched by the series of *c* and *z*[•] ions, indicating unambiguous assignment of the K15Me3 modification. Both peptides have N22 deamidation that are commonly generated during peptide synthesis or sample handling steps. The detailed fragment ions assignment of the supplemental activation ETD-MS/MS spectrum of the unmodified and methylated peptide by Mascot search engine version 2.4 was shown in Supplementary Tables 1 and 2. As a mixture of unmodified, oxidized, deamidated, and/or K15-trimethylated peptides were generated in the *in-vitro* methylation process, it is impractical to use extracted ion chromatogram method to integrate peak areas of various modified peptides for label-free quantitation. Spectral counting method was used to determine the K15-trimethylation efficiency by the Ezh2. We counted all MS/MS spectra matched to the peptides with no modification, methionine oxidation, deamidation and/or trimethylation. In total, there are 81 MS/MS spectra matched to the peptides. Among them, 31 MS/MS spectra were identified with trimethylation. In summary, the methylation efficiency of the Ezh2 on this peptide is determined to be 37% (details see corresponding Source Data file).

Supplementary Figure 7

Tri-methylation of talin1 at Lys2454 is critical for binding to F-actin.

(a) Recombinant talin1(2300-2451) containing C-terminal actin-binding site and dimerization domain was purified and incubated with F-actin. Binding of the recombinant talin1(2300-2451) (wild-type or with mutations at position K2454) to F-actin was determined using the actin-co-sedimentation assays with 4 μM of talin1(2300-2451) and indicated amounts of F-actin; talin1 and actin (1 μM) input (In), co-sedimented talin and actin (Pellet), remaining talin1(2300-2451) in supernatant (Supernatant). Representative images from 1 of 3 independent experiments are shown. (b) Quantitative analysis of the binding of talin1 mutants to various concentrations of F-actin was determined by actin co-sedimentation assay. Means ± s.d. of 3-5 independent experiments are shown, talin1(2300-2541)-K2454 (black squares), talin1(2300-2541)-K2454Q (blue diamonds), talin1(2300-2541)-K2454F (red triangles), talin1(2300-2541)-D2447N (filled purple circle), talin1(2300-2541)-V2444D (open green circle). (Two-way ANOVA with replication). *** $P=1.8 \times 10^{-8}$ (top), 1.2×10^{-11} (bottom). (c) The dissociation rate of F-actin from various talin1(2300-2451) was analyzed using Surface plasmon resonance (SPR) measurement as described in materials and methods. Dissociation response for wild-type talin1(2300-2541)-K2454/F-actin at 4, 20 and 40 μM was analyzed, and globally fit rate of dissociation was $k_d=1.4 \times 10^{-5} \text{ s}^{-1}$ (second panel from top). Dissociation response for talin1(2300-2541)-K2454Q/F-actin at 4, 20 and 40 μM was analyzed, and globally fit rate of dissociation was $k_d=4.14 \times 10^{-6} \text{ s}^{-1}$ ($P < 0.0001$ against wild-type talin1(2300-2541)-K2454) (third panel from top). Dissociation response for talin1(2300-2541)-K2454F/F-actin complex was not detectable at 4 μM, but consistently measurable at 10, 20 and 40 μM, and globally fit rate of dissociation was $k_d=1.4$

$\times 10^{-5} \text{ s}^{-1}$ ($P=0.09$ against wild-type talin1(2300-2541)-K2454) (bottom panel). A typical sensorgram showing overlay responses of complex concentrations from initial capturing followed by dissociation (top panel). The initial capturing phase of other graphs was shortened to improve clarity. **(d)** Biotin labeled dimeric talin1 peptides (aa2450-2461) containing tri-methylated (open bars) or unmodified K2454 (filled bars) were incubated with various concentrations of F-actin and analyzed for their co-sedimentation as described in **Fig. 7d** except that the co-sedimentated peptides were visualized on dot blots by streptavidin-HRP. Error bars represent the mean $\pm$ s.d. of 3 independent experiments. $*P=0.026$ (left), 0.013 (right). **(e-g)** The talin1-K2454me3 antibody was generated by immunizing rabbits with (Ac-SEAMK(Me)3RLQAAG)2-K-C-amide branched peptides conjugated to carrier protein followed by affinity depletion and purification (YenZym antibodies LLC.). The specificity of this talin1-K2454me3 antibody was verified by dot-blotting of non-methylated and tri-methylated peptides **(e)**, immunoblotting (IB) of endogenous talin1 (Endo-talin1) using tri-methylated talin1-K2454 peptide-blocking ($1 \mu\text{g/ml}$) as negative controls **(f)**, and recombinant GFP-talin1 fusion protein using GFP-talin1-2454 mutants as negative controls **(g)**.

Supplementary Figure 8

Ezh2 regulates integrin-dependent, but not integrin-independent, adhesion and migration of leukocytes.

(a) Cervical lymph node cells were prepared from *Ezh2^{fl/fl}* and *Ezh2^{fl/fl} Cd11c-Cre* mice 24h after FITC painting onto the dorsum of ears. CD11c⁺FITC⁺ DCs were analyzed by FACS. Representative plots (left) and percentages of FITC-positive DCs from 7 pairs of mice (right) are shown. Red line indicates the average percentages of FITC⁺ cells in control and Ezh2-deficient mice. (b) Control and Ezh2-deficient mature bone marrow derived DCs and BM neutrophils isolated from poly(I)-poly(C) treated *Ezh2^{fl/fl}* and *Ezh2^{fl/fl} Mx1-Cre* mice were plated on poly-L-lysine coated slides for 1 h and F-actin was visualized by phalloidin. F-actin (green), talin1 (red), DAPI (blue). Data shown are representative from 1 of the 2 independent experiments. Scale bars, 10 μ m. (c) Epidermal Langerhans cells (LCs) from control *Ezh2^{fl/fl}* and *Ezh2^{fl/fl} Cd11c-Cre* mice were identified by MHCII staining in steady-state and upon oxazolone stimulations. Representative images of more than 3 independent experiments are shown. Left to right, *** $p=6.6\times10^{-5}$, 8.5×10^{-5} . Scale bars, 100 μ m. (d) Migratory LCs (CD11c⁺MHCII^{hi}EpCAM⁺CD103⁺) in skin draining lymph nodes from control *Ezh2^{fl/fl}* and *Ezh2^{fl/fl} Cd11c-Cre* mice were determined by FACS and cell numbers were calculated. * $p=0.01$, ** $p=0.002$ (e) LCs as in c isolated from epidermis were plated on laminin coated slides for 2 h and identified by MHCII staining. The adhesion structures were determined by anti-talin1 antibody. Cell area was quantified from images. Thirty cells per mouse were scored. *** $p=1.3\times10^{-5}$. Scale bars, 5 μ m. (f) Various adhesion structures in control and Ezh2-deficient LCs were quantified according to the criteria specified in **Supplementary Fig. 4e**. Forty cells per mouse were scored. Error bars in the figures c-f represent mean $\pm$ s.e.m. of 3 independent experiments with 3 mice per group (two-tailed student's t-test with equal variance). Left to right, *** $p=1.8\times10^{-7}$, 7.0×10^{-6} , 1.5×10^{-5} . (g) Migration of control (filled bars) and Ezh2-deficient mature BMDCs (open bars) as defined in b was determined on surfaces coated with various concentrations of fibronectin. Time-lapse images were taken every 5 min for 2 h. Error bars in the figure represent mean $\pm$ s.e.m. of around 169-300 cells pooled from 2-3 independent experiments. *** $p=2.5\times10^{-18}$. (h) Migration of control (filled bars) and Ezh2-deficient DCs (open bars) was determined as described in g except some cells were treated with ROCK inhibitor Y27632 (Y27, 30 μ M) or myosin II inhibitor blebbistatin (ble, 10 μ M) while imaging. *** $p=3.6\times10^{-13}$. (i) Control (filled bars) and Ezh2-deficient mature BMDCs (open bars) were allowed to adhere to slides coated with indicated concentrations of fibronectin for 2 h. Cell areas were calculated using ImageJ. Error bars in h and i represent mean $\pm$ s.e.m. of around 200 cells pooled from 2 independent experiments. *** $p=3.6\times10^{-19}$. (j) Representative images from i are shown. Control (*Ezh2^{fl/fl}*) and Ezh2-deficient (*Ezh2^{Δ/Δ}*) mature BMDCs were cultured on surfaces coated with different concentrations of fibronectin (FN) in the presence or absence of Y27632. Focal adhesions were visualized by anti-paxillin staining (red), F-actin by Alexa Fluor 488® phalloidin and nucleus by DAPI (blue). Scale bars, 10 μ m.

Supplementary Tables

Monoisotopic mass of neutral peptide Mr(calc): 3355.7274
 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
 Variable modifications:
 N22 : Deamidated_N (N)
 30 : Biotin_C1 (C-term)
 C-term : Biotin_C1 (C-term)
 Ions Score: 66 Expect: 2.6e-008
 Matches : 120/502 fragment ions using 180 most intense peaks ([help](#))

#	b	b ⁺⁺	b ⁺	b ⁺⁺⁺	b ⁰	b ⁰⁺⁺	c	c ⁺⁺	Seq.	w	y	y ⁺⁺	y ⁺	y ⁺⁺⁺	y ⁰	y ⁰⁺⁺	z+1	z+1 ⁺⁺	z+2	z+2 ⁺⁺	#
1	100.0757	50.5415					117.1022	59.0548	V												29
2	171.1128	86.0600					188.1394	94.5733	A		3257.6664	1629.3368	3240.6398	1620.8236	3239.6558	1620.3315	3241.6477	1621.3275	3242.6555	1621.8314	28
3	331.1435	166.0754					348.1700	174.5886	C	3080.6092	3186.6293	1593.8183	3169.6027	1585.3050	3168.6187	1584.8130	3170.6105	1585.8089	3171.6184	1586.3128	27
4	459.2384	230.1228	442.2119	221.6096			476.2650	238.6361	K	2952.5142	3026.5986	1513.8029	3009.5721	1505.2897	3008.5881	1504.7977	3010.5799	1505.7936	3011.5877	1506.2975	26
5	558.3068	279.6571	541.2803	271.1438			575.3334	288.1703	V	2867.4615	2898.5037	1449.7555	2881.4771	1441.2422	2880.4931	1440.7502	2882.4849	1441.7461	2883.4928	1442.2500	25
6	686.4018	343.7045	669.3752	335.1913			703.4283	352.2178	K	2725.3508	2799.4352	1400.2213	2782.4087	1391.7080	2781.4247	1391.2160	2783.4165	1392.2119	2784.4243	1392.7158	24
7	757.4389	379.2231	740.4124	370.7098			774.4655	387.7364	A		2671.3403	1336.1738	2654.3137	1327.6605	2653.3297	1327.1685	2655.3216	1328.1644	2656.3294	1328.6683	23
8	872.4658	436.7366	855.4393	428.2233	854.4553	427.7313	889.4924	445.2498	D	2539.2868	2600.3032	1300.6552	2583.2766	1292.1419	2582.2926	1291.6499	2584.2844	1292.6459	2585.2923	1293.1498	22
9	1000.5244	500.7659	983.4979	492.2526	982.5139	491.7606	1017.5510	509.2791	Q	2411.2282	2485.2762	1243.1417	2468.2497	1234.6285	2467.2657	1234.1365	2469.2575	1235.1324	2470.2653	1235.6363	21
10	1115.5514	558.2793	1098.5248	549.7660	1097.5408	549.2740	1132.5779	566.7926	D	2296.2013	2357.2176	1179.1125	2340.1911	1170.5992	2339.2071	1170.1072	2341.1989	1171.1031	2342.2067	1171.6070	20
11	1202.5834	601.7953	1185.5569	593.2821	1184.5728	592.7901	1219.6099	610.3086	S	2209.1692	2242.1907	1121.5990	2225.1642	1113.0857	2224.1801	1112.5937	2226.1720	1113.5896	2227.1798	1114.0935	19
12	1331.6260	666.3166	1314.5994	657.8034	1313.6154	657.3114	1348.6525	674.8299	E	2080.1266	2155.1587	1078.0830	2138.1321	1069.5697	2137.1481	1069.0777	2139.1399	1070.0736	2140.1478	1070.5775	18
13	1402.6631	701.8352	1385.6366	693.3219	1384.6525	692.8299	1419.6897	710.3485	A		2026.1161	1013.5617	2009.0895	1005.0484	2008.1055	1004.5564	2010.0974	1005.5523	2011.1052	1006.0562	17
14	1533.7036	767.3554	1516.6770	758.8422	1515.6930	758.3502	1550.7301	775.8687	M	1878.0490	1955.0790	978.0431	1938.0524	969.5298	1937.0684	969.0378	1939.0602	970.0338	1940.0681	970.5377	16
15	1661.7986	831.4029	1644.7720	822.8896	1643.7880	822.3976	1678.8251	839.9162	K	1749.9541	1824.0385	912.5229	1807.0119	904.0096	1806.0279	903.5176	1808.0198	904.5135	1809.0276	905.0174	15
16	1817.8997	909.4535	1800.8731	900.9402	1799.8891	900.4482	1834.9262	917.9667	R	1593.8530	1695.9435	848.4754	1678.9170	839.9621	1677.9330	839.4701	1679.9248	840.4660	1680.9326	840.9699	14
17	1930.9837	965.9955	1913.9572	957.4822	1912.9732	956.9902	1948.0103	974.5088	L	1480.7689	1539.8424	770.4248	1522.8159	761.9116	1521.8318	761.4196	1523.8237	762.4155	1524.8315	762.9194	13
18	2059.0423	1030.0248	2042.0158	1021.5115	2041.0317	1021.0195	2076.0689	1038.5381	Q	1352.7103	1426.7583	713.8828	1409.7318	705.3695	1408.7478	704.8775	1410.7396	705.8734	1411.7474	706.3774	12
19	2130.0794	1065.5433	2113.0529	1057.0301	2112.0689	1056.5381	2147.1060	1074.0566	A		1298.6998	649.8535	1281.6732	641.3402	1280.6892	640.8482	1282.6810	641.8442	1283.6889	642.3481	11
20	2201.1165	1101.0619	2184.0900	1092.5486	2183.1060	1092.0566	2218.1431	1109.5752	A		1227.6627	614.3350	1210.6361	605.8217	1209.6521	605.3297	1211.6439	606.3256	1212.6518	606.8295	10
21	2258.1380	1129.5726	2241.1114	1121.0594	2240.1274	1120.5674	2275.1645	1138.0859	G		1156.6255	578.8164	1139.5990	570.3031	1138.6150	569.8111	1140.6068	570.8070	1141.6146	571.3110	9
22	2373.1649	1187.0861	2356.1384	1178.5728	2355.1544	1178.0808	2390.1915	1195.5994	N	1038.5877	1099.6041	550.3057	1082.5775	541.7924	1081.5935	541.3004	1083.5854	542.2963	1084.5932	542.8002	8
23	2444.2021	1222.6047	2427.1755	1214.0914	2426.1915	1213.5994	2461.2286	1231.1179	A		984.5771	492.7922	967.5506	484.2789	966.5666	483.7869	968.5584	484.7828	969.5662	485.2868	7
24	2543.2705	1272.1389	2526.2439	1263.6256	2525.2599	1263.1336	2560.2970	1280.6521	V	882.4978	913.5400	457.2736	896.5135	448.7604	895.5295	448.2684	897.5213	449.2643	898.5291	449.7682	6
25	2671.3654	1336.1864	2654.3389	1327.6731	2653.3549	1327.1811	2688.3920	1344.6996	K	740.3872	814.4716	407.7394	797.4451	399.2262	796.4610	398.7342	798.4529	399.7301	799.4607	400.2340	5
26	2827.4665	1414.2369	2810.4400	1405.7236	2809.4560	1405.2316	2844.4931	1422.7502	R	584.2861	686.3766	343.6920	669.3501	335.1787	668.3661	334.6867	670.3579	335.6826	671.3657	336.1865	4
27	2898.5037	1449.7555	2881.4771	1441.2422	2880.4931	1440.7502	2915.5302	1458.2687	A		530.2755	265.6414	513.2490	257.1281	512.2650	256.6361	514.2568	257.6320	515.2646	258.1360	3
28	2985.5357	1493.2715	2968.5091	1484.7582	2967.5251	1484.2662	3002.5622	1501.7848	S	426.2170	459.2384	230.1228	442.2119	221.6096	441.2279	221.1176	443.2197	222.1135	444.2275	222.6174	2
29									K	298.1220	372.2064	186.6068	355.1798	178.0936			356.1877	178.5975	357.1955	179.1014	1

Supplementary Table 1 Detailed fragment ions assignment of the unmodified peptide.

The detailed fragment ions assignment of the supplemental activation ETD-MS/MS spectrum of the unmodified peptide by Mascot search engine version 2.4. Red font shows the identified fragment ions. Extensive *c* and *z*⁺ ions, and some *b* and *y*-ions were identified from the ETD-MS/MS spectrum.

Monoisotopic mass of neutral peptide Mr(calc): 3397.7744
 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
 Variable modifications:
 K15 : Methyl3k (K)
 N22 : Deamidated_N (N)
 30 : Biotin_C1 (C-term)
 C-term : Biotin_C1 (C-term)
 Ions Score: 62 Expect: 2.4e-007
 Matches : 112/502 fragment ions using 178 most intense peaks ([help](#))

#	b	b ⁺⁺	b [*]	b ⁺⁺⁺	b ⁰	b ⁰⁺⁺	c	c ⁺⁺	Seq.	w	y	y ⁺⁺	y [*]	y ⁺⁺⁺	y ⁰	y ⁰⁺⁺	z+1	z+1 ⁺⁺	z+2	z+2 ⁺⁺	#
1	100.0757	50.5415					117.1022	59.0548	V												29
2	171.1128	86.0600					188.1394	94.5733	A		3299.7133	1650.3603	3282.6868	1641.8470	3281.7028	1641.3550	3283.6946	1642.3509	3284.7024	1642.8549	28
3	331.1435	166.0754					348.1700	174.5886	C	3122.6561	3228.6762	1614.8417	3211.6497	1606.3285	3210.6657	1605.8365	3212.6575	1606.8324	3213.6653	1607.3363	27
4	459.2384	230.1228	442.2119	221.6096			476.2650	238.6361	K	2994.5612	3068.6456	1534.8264	3051.6190	1526.3131	3050.6350	1525.8211	3052.6268	1526.8171	3053.6347	1527.3210	26
5	558.3068	279.6571	541.2803	271.1438			575.3334	288.1703	V	2909.5084	2940.5506	1470.7789	2923.5241	1462.2657	2922.5400	1461.7737	2924.5319	1462.7696	2925.5397	1463.2735	25
6	686.4018	343.7045	669.3752	335.1913			703.4283	352.2178	K	2767.3978	2841.4822	1421.2447	2824.4556	1412.7315	2823.4716	1412.2395	2825.4635	1413.2354	2826.4713	1413.7393	24
7	757.4389	379.2231	740.4124	370.7098			774.4655	387.7364	A		2713.3872	1357.1973	2696.3607	1348.6840	2695.3767	1348.1920	2697.3685	1349.1879	2698.3763	1349.6918	23
8	872.4658	436.7366	855.4393	428.2233	854.4553	427.7313	889.4924	445.2498	D	2581.3337	2642.3501	1321.6787	2625.3236	1313.1654	2624.3396	1312.6734	2626.3314	1313.6693	2627.3392	1314.1732	22
9	1000.5244	500.7659	983.4979	492.2526	982.5139	491.7606	1017.5510	509.2791	Q	2453.2752	2527.3232	1264.1652	2510.2966	1255.6519	2509.3126	1255.1599	2511.3044	1256.1559	2512.3123	1256.6598	21
10	1115.5514	558.2793	1098.5248	549.7660	1097.5408	549.2740	1132.5779	566.7926	D	2338.2482	2399.2646	1200.1359	2382.2380	1191.6227	2381.2540	1191.1307	2383.2459	1192.1266	2384.2537	1192.6305	20
11	1202.5834	601.7953	1185.5569	593.2821	1184.5728	592.7901	1219.6099	610.3086	S	2251.2162	2284.2377	1142.6225	2267.2111	1134.1092	2266.2271	1133.6172	2268.2189	1134.6131	2269.2268	1135.1170	19
12	1331.6260	666.3166	1314.5994	657.8034	1313.6154	657.3114	1348.6525	674.8299	E	2122.1736	2197.2056	1099.1064	2180.1791	1090.5932	2179.1951	1090.1012	2181.1869	1091.0971	2182.1947	1091.6010	18
13	1402.6631	701.8352	1385.6366	693.3219	1384.6525	692.8299	1419.6897	710.3485	A		2068.1630	1034.5852	2051.1365	1026.0719	2050.1525	1025.5799	2052.1443	1026.5758	2053.1521	1027.0797	17
14	1533.7036	767.3554	1516.6770	758.8422	1515.6930	758.3502	1550.7301	775.8687	M	1920.0960	1997.1259	999.0666	1980.0994	990.5533	1979.1154	990.0613	1981.1072	991.0572	1982.1150	991.5611	16
15	1703.8455	852.4264	1686.8190	843.9131	1685.8349	843.4211	1720.8721	860.9397	K	1749.9541	1866.0854	933.5464	1849.0589	925.0331	1848.0749	924.5411	1850.0667	925.5370	1851.0745	926.0409	15
16	1859.9466	930.4769	1842.9201	921.9637	1841.9360	921.4717	1876.9732	938.9902	R	1593.8530	1695.9435	848.4754	1678.9170	839.9621	1677.9330	839.4701	1679.9248	840.4660	1680.9326	840.9699	14
17	1973.0307	987.0190	1956.0041	978.5057	1955.0201	978.0137	1990.0572	995.5323	L	1480.7689	1539.8424	770.4248	1522.8159	761.9116	1521.8318	761.4196	1523.8237	762.4155	1524.8315	762.9194	13
18	2101.0893	1051.0483	2084.0627	1042.5350	2083.0787	1042.0430	2118.1158	1059.5615	Q	1352.7103	1426.7583	713.8828	1409.7318	705.3695	1408.7478	704.8775	1410.7396	705.8734	1411.7474	706.3774	12
19	2172.1264	1086.5668	2155.0998	1078.0535	2154.1158	1077.5615	2189.1529	1095.0801	A		1298.6998	649.8535	1281.6732	641.3402	1280.6892	640.8482	1282.6810	641.8442	1283.6889	642.3481	11
20	2243.1635	1122.0854	2226.1369	1113.5721	2225.1529	1113.0801	2260.1900	1130.5987	A		1227.6627	614.3350	1210.6361	605.8217	1209.6521	605.3297	1211.6439	606.3256	1212.6518	606.8295	10
21	2300.1849	1150.5961	2283.1584	1142.0828	2282.1744	1141.5908	2317.2115	1159.1094	G		1156.6255	578.8164	1139.5990	570.3031	1138.6150	569.8111	1140.6068	570.8070	1141.6146	571.3110	9
22	2415.2119	1208.1096	2398.1853	1199.5963	2397.2013	1199.1043	2432.2384	1216.6229	N	1038.5877	1099.6041	550.3057	1082.5775	541.7924	1081.5935	541.3004	1083.5854	542.2963	1084.5932	542.8002	8
23	2486.2490	1243.6281	2469.2225	1235.1149	2468.2384	1234.6229	2503.2756	1252.1414	A		984.5771	492.7922	967.5506	484.2789	966.5666	483.7869	968.5584	484.7828	969.5662	485.2868	7
24	2585.3174	1293.1623	2568.2909	1284.6491	2567.3069	1284.1571	2602.3440	1301.6756	V	882.4978	913.5400	457.2736	896.5135	448.7604	895.5295	448.2684	897.5213	449.2643	898.5291	449.7682	6
25	2713.4124	1357.2098	2696.3858	1348.6966	2695.4018	1348.2045	2730.4389	1365.7231	K	740.3872	814.4716	407.7394	797.4451	399.2262	796.4610	398.7342	798.4529	399.7301	799.4607	400.2340	5
26	2869.5135	1435.2604	2852.4869	1426.7471	2851.5029	1426.2551	2886.5400	1443.7737	R	584.2861	686.3766	343.6920	669.3501	335.1787	668.3661	334.6867	670.3579	335.6826	671.3657	336.1865	4
27	2940.5506	1470.7789	2923.5241	1462.2657	2922.5400	1461.7737	2957.5772	1479.2922	A		530.2755	265.6414	513.2490	257.1281	512.2650	256.6361	514.2568	257.6320	515.2646	258.1360	3
28	3027.5826	1514.2950	3010.5561	1505.7817	3009.5721	1505.2897	3044.6092	1522.8082	S	426.2170	459.2384	230.1228	442.2119	221.6096	441.2279	221.1176	443.2197	222.1135	444.2275	222.6174	2
29									K	298.1220	372.2064	186.6068	355.1798	178.0936			356.1877	178.5975	357.1955	179.1014	1

Supplementary Table 2 Detailed fragment ions assignment of the methylated peptide.

The detailed fragment ions assignment of the supplemental activation ETD-MS/MS spectrum of the K15Me modified peptide by Mascot search engine version 2.