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Antagonistic actions of Rcor proteins regulate LSD1 activity and cellular differentiation

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Lysine-specific demethylase 1 (LSD1) demethylates nucleosomal histone H3 lysine 4 (H3K4) residues in collaboration with the corepressor CoREST/REST corepressor 1 (Rcor1) and regulates cell fates by epigenetically repressing gene targets. The balanced regulation of this demethylase, if any, is however unknown. We now demonstrate the actions of two other Rcor paralogs, Rcor2 and Rcor3, in regulating LSD1 enzymatic activity and biological function in hematopoietic cells. All three Rcor proteins interact with LSD1 and with the erythro-megakaryocytic transcription factor growth factor independence (Gfi)1b; however, whereas Rcor2, like Rcor1, facilitates LSD1-mediated nucleosomal demethylation, Rcor3 competitively inhibits this process. Appending the SANT2 domain of Rcor1 to Rcor3 confers the ability to facilitate LSD1-mediated demethylation on the chimeric Rcor protein. Consistent with their biochemical activities, endogenous Rcor1, Rcor2, and LSD1 promote differentiation, whereas Rcor3 opposes these processes. Recruitment of Rcor3 to cognate gene targets by Gfi1b and LSD1 leads to inhibition of H3K4 demethylation of chromatin and transcriptional derepression of these loci. Remarkably, profound alterations in Rcor1/3 levels during erythroid versus megakaryocytic differentiation potentiate antagonistic outcomes. In mature erythroid cells, a strong upsurge in Rcor3 and a sharp decline in Rcor1 levels counteract LSD1/Rcor1/2-mediated differentiation. In contrast, the opposite changes in Rcor1/3 levels in megakaryocytes favor differentiation and likely maintain homeostasis between these lineages. Overall, our results identify Rcor3 as a natural inhibitor of LSD1 and highlight a dual mechanism of regulating the enzymatic activity and restraining the epigenetic impact of this robust demethylase during hematopoietic differentiation.

Lysine-specific demethylase 1 (LSD1; also known as Kdm1a, Aof2, and BHC110) removes dimethyl or monomethyl moieties from histone H3 lysine 4 (H3K4) residues on free histones (1), but requires facilitation from the corepressor CoREST/REST corepressor 1 (Rcor1) to catalyze this reaction on chromatin (nucleosomes) (2, 3). LSD1 also has been reported to demethylate H3K9 residues on chromatin in a context-dependent manner, but not in vitro (4, 5). Various experiments have delineated the roles of distinct LSD1 and Rcor1 domains in mediating interactions between them and/or in facilitating catalysis. Whereas the ~120-aa-long "linker" region of Rcor1 mediates interaction with LSD1 (3), either both SANT (Swi3, Ada2, N-CoR, and TFIIIB) domains (2) or only the SANT2 domain (3) have been identified as essential for facilitating catalysis. Biophysical studies subsequently revealed the "nanoscale clamp" formed by the LSD1/Rcor1 co-complex on nucleosomes (6, 7) and highlighted the contribution of the SANT2 domain in stabilizing this structure. The contributions of the SANT or other domains to the cellular functions of Rcor1 remain unknown, however.

LSD1 and Rcor1 are recruited by different transcription factors in diverse tissues and contexts. In hematopoietic cells, they associate with growth factor independence (Gfi)1 and Gfi1b and repress the majority of Gfi1b gene targets in erythroid cells (8). This duo also associates with Scl1/Tal1 (9, 10) and Bcl11A (11) in erythroid cells and mediates repression of their target genes. Therefore, LSD1 inhibition or conditional deletion diminishes or abolishes differentiation of normal hematopoietic stem and progenitor cells (12, 13), respectively. In contrast, the Rcor1 germline deletion was recently demonstrated to specifically abrogate erythroid differentiation (14). Intriguingly, LSD1 is overexpressed in leukemic stem cells, where it either sustains the oncogenic potential (15) or suppresses differentiation (16). These apparent anomalies may imply the existence of additional regulatory mechanisms for modulating LSD1 activity in specific cellular contexts.

Here we delineate the actions of two additional Rcor factors, Rcor2 and Rcor3, in regulating LSD1 activity and function. We further demonstrate the consequences of these processes on erythro-megakaryocytic differentiation and the actions of Rcor3 on cognate gene targets. Finally, we correlate the effects mediated by these factors with their endogenous expression in primary hematopoietic cells to account for their in vivo functions.

Results

LSD1 and Rcor1-3 were previously identified in a proteomic screen for Gfi1b-associated proteins (8). Subsequent amino acid sequence alignments of the Rcor proteins revealed conservation of most of the major domains (Elm2, SANT1, and linker) among them (Fig. S1*A*). Consistent with the presence of a conserved linker domain, Rcor2 and Rcor3, like Rcor1, associated with LSD1 and with both Gfi1b and Gfi1 (Fig. 1*A*), but not with the SNAG domain mutant of Gfi1b (P2A-Gfi1b), thus reaffirming the function of this domain in recruiting LSD1/Rcor factors to Gfi1/1b protein complexes (8, 17–19). The lack of interactions with an unrelated protein, BirA (Fig. 1*A*, *Bottom*), and with total mouse IgG (Fig. S1 *B* and *C*) further confirmed the specificity of the interactions among these proteins. Subsequently, association between the endogenous proteins in erythroid cells was confirmed by coelution of Gfi1b/LSD1/Rcor protein complexes by size-exclusion chromatography (gel filtration) (Fig. 1 *B* and *C*).

Significance

This work illustrates the dual regulation of the potent and widely expressed demethylase lysine-specific demethylase 1 (LSD1) by the antagonistic actions of stimulatory and inhibitory Rcor factors and their consequences on hematopoietic differentiation. Given the crucial role of LSD1 in diverse developmental contexts, including ES cells, multiple mammalian tissues, and oncogenesis, these results could potentially reflect the function of LSD1 and Rcor1-3 in multiple normal and aberrant developmental contexts. Moreover, these insights could be used to develop targeted strategies for controlling LSD1 activity in different diseases.

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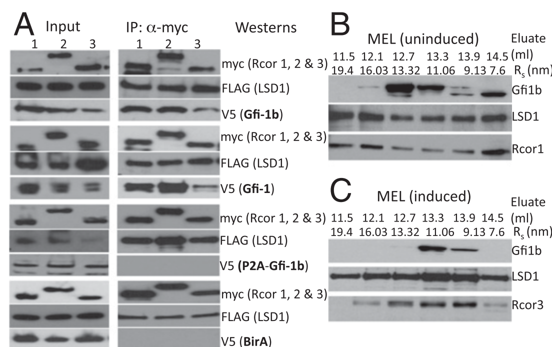

Fig. 1. Association of Rcor1-3 with LSD1 and Gfi proteins. (*A*) Western blot analysis of anti-myc immunoprecipitated material (IP: α-myc) alongside 10% of input extract (Input) from cells expressing combinations of epitope-tagged proteins. Shown are cellular extracts containing one of each type of epitope-tagged protein: myc (Rcor1, Rcor2, and Rcor3 in lanes 1, 2, and 3, respectively), FLAG (LSD1), and V5 (Gfi1b-top, Gfi1-second from top, P2A-Gfi1b-third from top and BirA bottom panels) in different combinations after immunoprecipitation and blotting as indicated. (*B* and *C*) Coelution of endogenous Gfi1b, LSD1, and Rcor1 from uninduced MEL cells (*B*) and Gfi1b, LSD1, and Rcor3 from induced MEL cells (*C*) in gel filtration assays. Elution volumes and Stokes radii (R_s) of eluates calculated on the basis of the elution volumes of prerun size standards (34) are indicated. Stokes radii of 11.06 nm and 7.6 nm correspond to complex sizes of ~700 kD and ~500 kD, respectively. Results represent one of three independent experiments.

Although LSD1, and to a lesser extent Rcor1/3, exhibited diffuse elution profiles, strong coelution was observed for Gfi1b and Rcor1/3 at volumes corresponding to complex sizes of 500–700 kDa in immature [uninduced murine erythroleukemia (MEL)] and mature (DMSO-induced MEL) erythroid cells exhibiting robust Rcor1 and Rcor3 expression, respectively. These results show that whereas LSD1 and Rcor1 form multiple complexes consistent with their known association with different proteins (9, 10), several of these overlap with Gfi1b complexes. In contrast, Rcor3 forms fewer complexes, most of which overlap significantly with those of Gfi1b.

Because sequence analysis also revealed the absence of the SANT2 domain in Rcor3 (Fig. S1*A*), suggesting functional differences among Rcor1, Rcor2, and Rcor3, we assessed the ability of these corepressors to regulate LSD1-mediated nucleosomal demethylation in vitro. Recombinant LSD1 and Rcor1-3 expressed and purified from bacteria (Fig. S2*A*) were tested for activity in these assays. As expected, LSD1 alone could demethylate dimeH3K4 in free histones, but needed facilitation from Rcor1 to demethylate nucleosomes (Fig. S2 *B* and *C*). As reported previously (20), Rcor2 exhibited similar activity as Rcor1, albeit with somewhat reduced efficiency (Fig. 2*A*). In contrast, Rcor3 failed to facilitate LSD1-mediated nucleosomal demethylation (Fig. 2*B*) and instead competitively inhibited Rcor1 activity in a dose-dependent manner (Fig. 2*C*). Meanwhile, methylation of H3K9 remained unchanged after the addition of LSD1 and Rcor1-3 to mononucleosomes, attesting to the specificity of LSD1 activity in vitro (Fig. 2 *A* and *C*).

Because the absence of the SANT2 domain is the major distinguishing feature between Rcor3 and Rcor1/2, we appended each of the Rcor1 SANT domains to Rcor3 downstream of the linker region and evaluated the activity of the resulting chimeric proteins (Fig. 2 *D* and *E*). Strikingly, addition of the SANT2 domain conferred the ability to facilitate LSD1 mediated nucleosomal demethylation on the chimeric Rcor3-SANT2 protein (Fig. 2*D*), but not that of the SANT1 domain (Fig. 2*E*). These results demonstrate inherent functional differences between the Rcor1 SANT domains even when compensated for positional differences in the Rcor backbone, and illustrate the structural basis for the inhibitory properties of Rcor3 relative to Rcor1/2.

We next interrogated the function of the Rcor proteins in modulating differentiation of the erythroid and megakaryocytic lineages specified by Gfi1b (21). To do so, we knocked down LSD1 and Rcor1-3 in primary hematopoietic cells (Fig. S3 and Fig. 3, respectively) and performed multiple assays to ascertain differentiation. Alterations in mRNA levels (Fig. 3 *B* and *G*), histochemical staining (benzidine and acetylcholine esterase for erythroid and megakaryocytic cells, respectively) (Fig. 3 *D* and *H*), and surface protein expression (by FACS analysis) (Fig. 3 *E* and *I*) in the manipulated cells revealed the impact of inhibiting these proteins. Consistent with previous studies (8, 12–14), inhibition of LSD1 (Fig. S3) and Rcor1 (Fig. 3) suppressed erythro-megakaryocytic differentiation in fetal liver hematopoietic progenitors transduced with the corresponding shRNAs and cultured in vitro. Likewise, inhibition of Rcor2 produced similar defects, demonstrating the normal role of this protein in promoting these lineages. In sharp contrast to these phenotypes, Rcor3 depletion produced the opposite result and augmented differentiation of these cells (Fig. 3).

To reiterate the antagonistic functions of Rcor1 and Rcor3 and eliminate the possibility of shRNA-mediated off-target effects, we performed reciprocal experiments involving ectopic overexpression of these proteins. As revealed by the same assays, these manipulations produced phenotypes that were the reverse of their corresponding knockdowns (Fig. 4). Rcor1 overexpression produced a moderate and consistent increase in differentiation, whereas Rcor3 overexpression significantly suppressed differentiation. Collectively, manipulation of LSD1 and Rcor1-3 expression demonstrates that LSD1 and Rcor1/2 stimulate erythro-megakaryocytic differentiation, whereas Rcor3 antagonizes it.

To ascertain the mechanistic consequences of altering Rcor3 levels on chromatin configuration, we performed chromatin immunoprecipitation (ChIP) experiments in Gfi1b target gene promoters in erythroid cells exhibiting differential Rcor1/3 expression. Rcor1 is expressed at high levels and Rcor3 is expressed at low/undetectable levels in immature erythroid cells, whereas the reverse expression pattern is observed in mature erythroid cells with high levels of Rcor3 and low levels of Rcor1 (8) (Fig. 5*A*). Gfi1b and LSD1 protein levels remain constant during these stages of erythroid differentiation. We interrogated selected Gfi1b promoters for their level of LSD1 and Rcor3 occupancy and corresponding dimeH3K4 levels in uninduced MEL cells (immature,

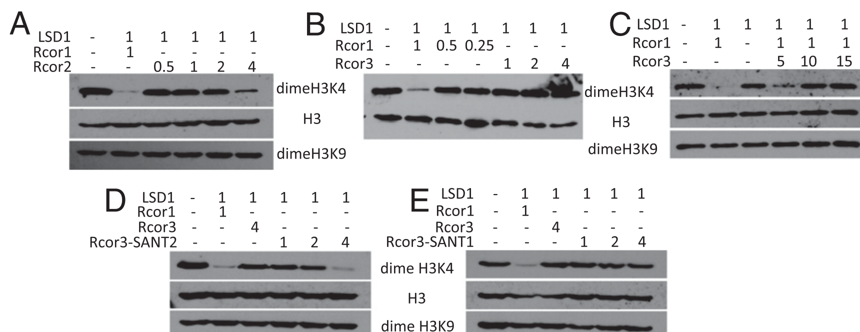

Fig. 2. Nucleosomal demethylation by LSD1 and Rcor proteins. Western blot analysis of di-meH3K4 (*Top*), total H3 (*Middle*), and dimeH3K9 (*Bottom*) levels in mononucleosomes (1 μg) treated with indicated amounts of different recombinant proteins. Shown are nucleosomes treated with LSD1, Rcor1, and Rcor2 (*A*); LSD1, Rcor1, and 1–4 μg of Rcor3 (*B*); LSD1, Rcor1, and 5–15 μg of Rcor3 (*C*); LSD1, Rcor1, Rcor3, and chimeric Rcor3-SANT2 (*D*); and LSD1, Rcor1, Rcor3, and chimeric or Rcor3-SANT1 (*E*). Results represent one of three independent experiments. Purification analysis of recombinant proteins is depicted in Fig. S2*A*.

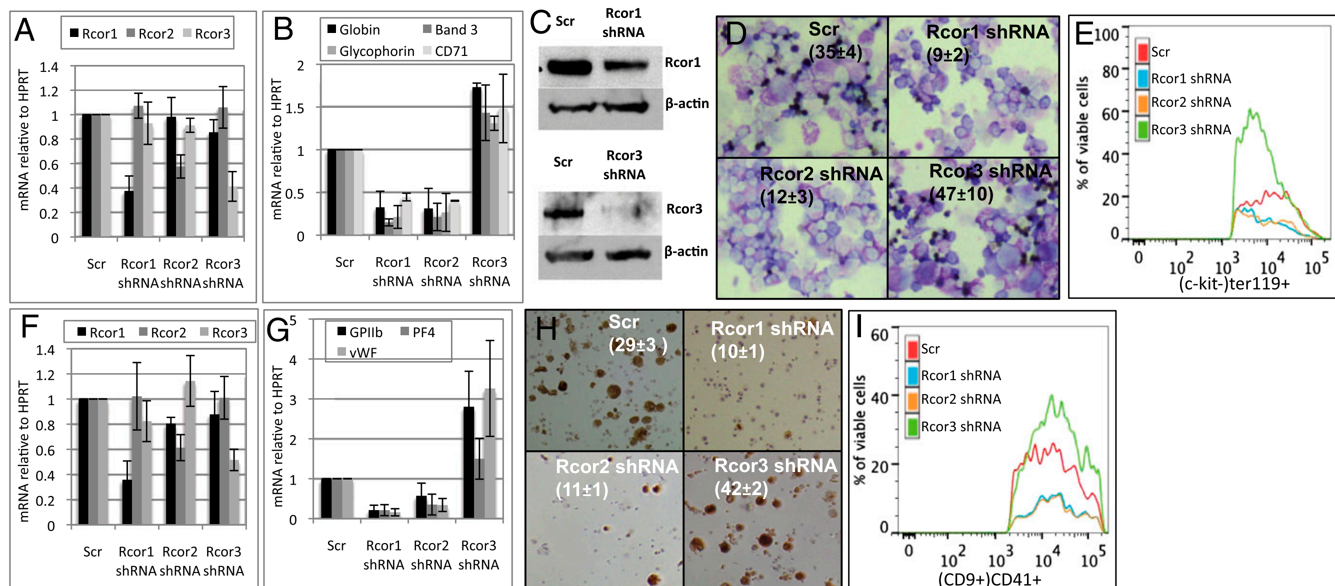

Fig. 3. Effect of Rcor1-3 inhibition on erythrocyte–megakaryocytic differentiation. (*A* and *B*) qPCR of Rcor1-3 (*A*) and erythroid differentiation markers β-major globin (Globin), erythrocyte band 3 (Band3), glycophorin A (glycophorin), and CD71 (transferrin receptor) (*B*) in E12.5 fetal liver cells transduced with Rcor1-3 shRNAs relative to scrambled shRNA controls (scr) and cultured in stem cell factor and erythropoietin. (*C*) Western blots for Rcor1, Rcor3, and β-actin in Rcor1/3-inhibited (shRNA) erythroid cells versus controls (scr); 40 μg of total protein was loaded in each lane. (*D*) Benzidine staining (for heme) of manipulated cells. (*E*) FACS histogram of surface marker ter119 in c-kit-/ter119^{+} gated cells from control and Rcor1-3–inhibited samples. (*F* and *G*) qPCR of Rcor1-3 (*F*) and megakaryocytic differentiation markers glycoprotein IIb (GPIIb), platelet factor 4 (PF4), and von Willebrand factor (vWF) (*G*) in fetal liver cells transduced with the indicated shRNAs and cultured in IL-3 and thrombopoietin. (*H*) Acetylcholine esterase staining of manipulated cells. (*I*) FACS analysis of megakaryocyte surface marker CD41/GPIIb in CD9^{+}CD41^{+} gated cells manipulated as indicated. All qPCR results represent the averages and SDs (error bars) of three independent experiments. Other results depict one of three representative experiments. In *D* and *H*, mean ± SD of positively staining cells as percent of total from three independent experiments is indicated in parentheses for histochemically stained cells.

corresponding to the proerythroblast stage) and induced cells (mature, representing the orthochromatic erythroblast stage). Consistent with elevated Rcor3 protein levels in induced versus uninduced MEL cells (Fig. 5*A*), we found increases in both the association of this protein with Gfi1b in solution (Fig. 5*B*) and on chromatin at defined loci (Fig. 5 *C* and *D*). This increase in Rcor3 levels in combination with a marginal decrease in LSD1 enrichment (possibly related to the reduced affinity of LSD1/Rcor3 complexes for nucleosomes owing to a lack of the SANT2 hook on Rcor3) led to a significant overall increase (threefold to fivefold) in dimethyl H3K4 levels at these loci in the induced/mature cells (Fig. 5*D*).

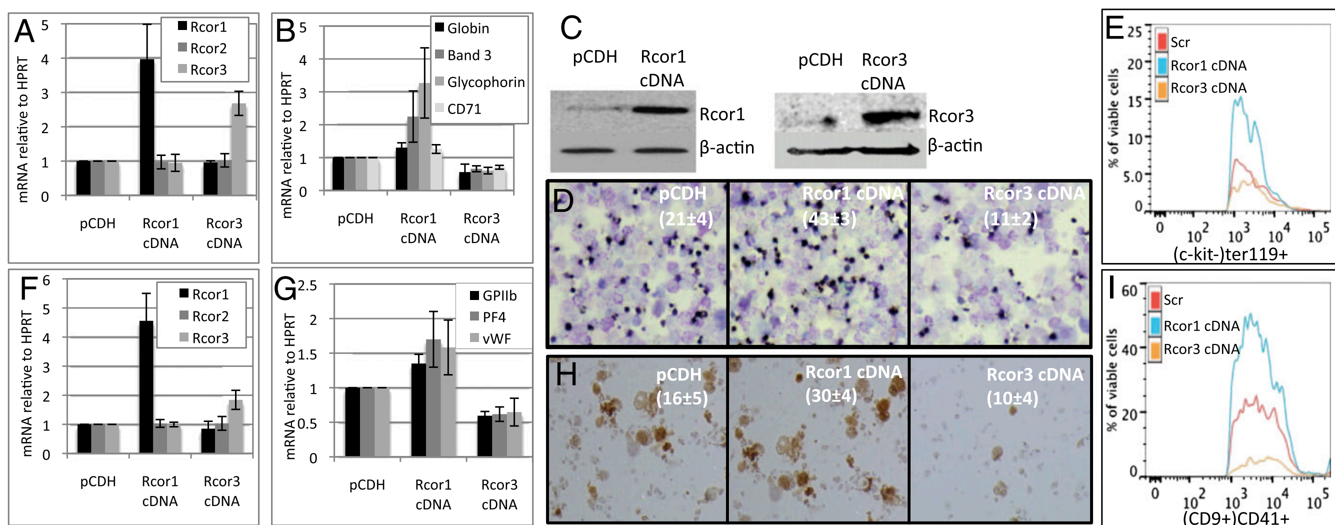

Fig. 4. Effect of ectopic Rcor1/3 expression on differentiation. (*A* and *B*) qPCR of Rcor1-3 (*A*) and erythroid differentiation markers (*B*) (as in Fig. 3) in cells overexpressing (cDNA) Rcor1/3 versus controls (pCDH). (*C*) Western blots of Rcor1, Rcor3, and β-actin in manipulated cells; 20 μg of total protein was loaded in each lane. (*D*) Benzidine staining of manipulated cells. (*E*) FACS histogram of surface marker ter119 in c-kit-ter119^{+} gated control and overexpressing cells. (*F* and *G*) qPCR of Rcor1-3 levels (*F*) and megakaryocytic differentiation markers (*G*) in manipulated cells. (*H*) Acetylcholine esterase staining of manipulated cells. (*I*) FACS analysis of CD41 in CD9^{+}CD41^{+} gated manipulated cells. Average or representative values of three experiments are shown. In *D* and *H*, mean ± SD of positive cells as a percentage of the total from three independent experiments is indicated in parentheses.

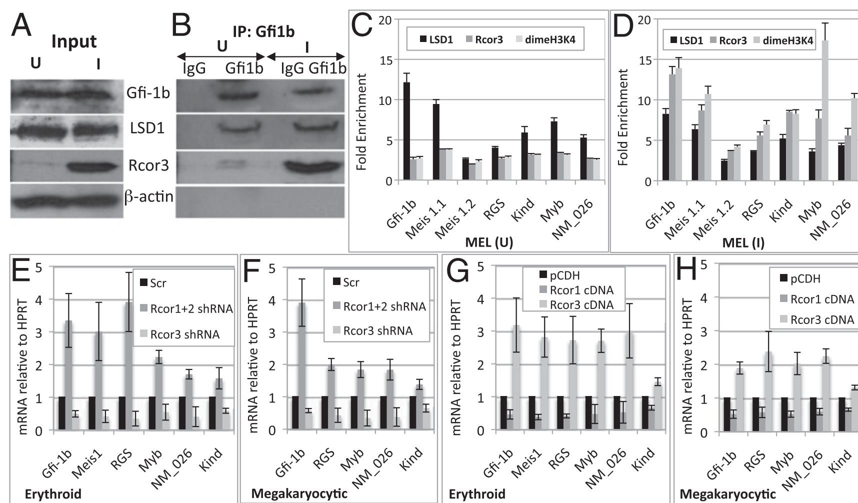

Fig. 5. Effect of Rcor3 on chromatin and gene expression. (*A*) Western blots of Gfi1b, LSD1, Rcor3, and β-actin in uninduced (U) and induced (I) MEL cells (representing 10% of the input for *B*). (*B*) Coimmunoprecipitation of endogenous LSD1 and Rcor3 by Gfi1b. (*C* and *D*) Relative enrichment of LSD1, Rcor3, and dimethyl H3K4 levels at Gfi1b target promoters Gfi1b, Meis1.1, and Meis1.2 promoter segments (30), c-myb, NM_026543, Rgs18, and kindlin3 (8) in uninduced (*C*) and induced (*D*) MEL cells. Enrichment was quantified relative to control IgG treated chromatin and to the IgH switch μ locus. (*E*–*H*) Expression of gene targets in Rcor1-3– inhibited primary erythroid (*E*) and megakaryocytic (*F*) cells and in Rcor1/3-overexpressing erythroid (*G*) and megakaryocytic (*H*) cells. Mean ± SD (error bars) of three independent experiments are shown.

To determine the effects of altering Rcor1-3 levels on ensuing target gene expression resulting from alterations in chromatin structure and activity at the corresponding loci, we monitored their mRNA levels in Rcor1-3–manipulated primary cells. As predicted, Rcor1/2 inhibition increased, but Rcor3 inhibition decreased, the expression of these targets (Fig. 5 *E* and *F*). The reverse pattern was observed on Rcor1/3 overexpression in both lineages (Fig. 5 *G* and *H*).

To establish LSD1-dependent recruitment of Rcor3 to gene targets, we evaluated the relative enrichment of Gfi1b, LSD1, and Rcor3 on cognate promoters in control and LSD1-depleted induced MEL cells (Fig. 6). Because Gfi1b binds directly to its DNA recognition element, we found no significant difference in the relative enrichment of Gfi1b at these loci between control and LSD1-depleted cells (Fig. 6 *B* and *C*). In contrast, enrichment of both LSD1 and Rcor3 was drastically reduced in LSD1-inhibited cells relative to controls. This reduction in Rcor3 enrichment in LSD1 knockdown cells occurred despite unchanged (Rcor3) protein levels (Fig. 6*A*) and was directly proportional to the level of LSD1 enrichment at these loci (Fig. 6 *B* and *C*). This result illustrates that recruitment of Rcor3 to chromatin is dependent on, and limited by, LSD1. This experiment confirms that the tethering of these proteins to DNA/chromatin follows the order Gfi1b-LSD1-Rcor3 and is entirely consistent with previous studies demonstrating a similar associative protein sequence among Gfi1b, LSD1, and Rcor1 (8).

Because Rcor1 is known to function only by modulating LSD1 activity, but not vice versa (4, 5), we interrogated the functional interdependence of these proteins in cells. We compared the consequences of inhibiting both LSD1 and Rcor1 relative to inhibiting the individual proteins in primary erythro-megakaryocytic cells. Dual inhibition of LSD1 and Rcor1 produced no additional suppression of differentiation compared with that obtained with the individual proteins, especially LSD1, alone (Fig. 6 *D* and *E*). The lack of a cumulative phenotype after inhibition of LSD1 and Rcor1 strongly suggests functional codependence between these factors and their use in the same process(es). Given that the extent of differentiation was essentially limited by the level/activity of LSD1, this result demonstrates that Rcor1 exerts its effects primarily via LSD1.

Because the relative concentrations of Rcor1/2 versus Rcor3 likely dictate the effective enzymatic activity of LSD1 in any given cell, we investigated Rcor1-3 expression during erythro-megakaryocytic differentiation. LSD1 and Rcor1-3 message and protein levels were determined in E14.5 fetal liver cells that had been FACS-sorted for CD71 and ter119 expression (22), which allowed separation of the erythroid cells into immature ($CD71^{hi}ter119^{-}$), intermediate ($CD71^{hi}ter119^{+}$), and mature ($CD71^{lo}ter119^{+}$) populations (Fig. S4*A*). LSD1 message and protein levels were fairly robust and uniform in all three populations (Fig. 7), as were Rcor2 mRNA levels. However, Rcor1 mRNA and protein levels declined sharply (~8-fold), and Rcor3 levels surged dramatically (~30- to 40-fold) in the mature cell population (Fig. 7 *A* and *B*). This shift in Rcor1/3 levels during erythroid maturation (observed previously in differentiating MEL cells; Fig. 5*A*) should produce a concomitant decline in LSD1 enzymatic activity in mature cells despite unchanged

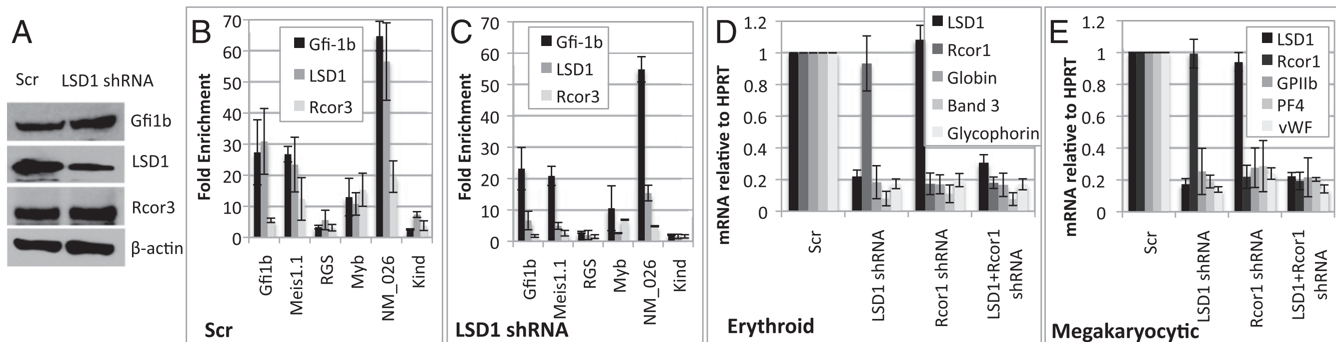

Fig. 6. Dependence of Rcor protein function on LSD1. (*A*–*C*) Endogenous Gfi1b, LSD1, Rcor3, and β-actin protein expression (*A*) and enrichment at target gene loci in scrambled (scr; *B*) and LSD1-inhibited (LSD1 shRNA; *C*) induced MEL cells. (*D* and *E*) qPCR analysis of LSD1, Rcor1, and erythroid mRNAs (*D*) or megakaryocytic mRNAs (*E*) in fetal liver cells transduced with LSD1, Rcor1, or LSD1 and Rcor1 shRNAs. Mean ± SD (error bars) of three independent experiments are shown for *B*–*E*.

expression, and extinguish the differentiation program driven by LSD1-Rcor1/2 (Fig. S4B).

In contrast to erythroid cells, during megakaryocytic differentiation of E12.5 fetal liver cells in vitro (as inferred from GPIIb levels and visual inspection), message levels of Rcor1/3 exhibited the reverse trend. Message levels of Rcor1 showed a steady increase and those of Rcor3 a sharp drop, whereas those of LSD1 and Rcor2 remained unchanged (Fig. 7C). Because megakaryocytic lineage cells are relatively rare in fetal livers and cannot be reliably sorted out at this developmental stage, we were unable to assess the expression pattern of these factors during megakaryocytic differentiation in vivo. Nonetheless, the divergent endogenous expression profiles of Rcor1/3 during erythro-megakaryocytic differentiation, in conjunction with the phenotypes produced by manipulating their levels in these cells, indicate that they produce opposite outcomes in the two lineages.

Overall, our present results and those from previous reports (2, 3, 8, 11–13, 20) establish that Rcor1 and Rcor2 facilitate LSD1-mediated demethylation to collaboratively repress target genes and promote erythro-megakaryocytic differentiation. In contrast, Rcor3 competitively inhibits LSD1 activity and antagonizes differentiation. Thus, with LSD1 levels remaining constant, relative expression levels of Rcor1-3 determine the extent of differentiation of these cells, and likely other cells as well.

Discussion

This study demonstrates how antagonistic regulation of LSD1 activity by Rcor1/2 versus Rcor3 determines its effective H3K4 demethylase activity in specific chromatin and cellular contexts, thereby modulating gene expression and arbitrating cell fate (Fig. S4B). These observations, although specifically documented for Gfi1b targets and erythro-megakaryocytic differentiation, are relevant to, and critical for, determining LSD1 function in diverse loci and cells. First, in addition to Gfi1/1b, LSD1 and Rcor1 are harnessed by other hematopoietic transcription factors, including Scl1/Tal1, Bcl11A, and Sall4 (8, 10, 11, 23), to repress transcription of their gene targets. Thus, coordinated regulation of LSD1 by Rcor1-3 likely ensures multilineage differentiation and appropriate homeostasis by regulating the gene targets of these and other, as-yet unidentified, factors during hematopoietic development.

Second, LSD1 is vital for the development of many mammalian organs and tissues, as evidenced by complete and tissue-specific knockout/knockdown studies that produced either early (peri-implantation) embryonic lethality or severe developmental defects, respectively (12, 13, 24). In contrast, the role of Rcor1-3 in mammalian development remains partially documented. Recent germline deletion of Rcor1 confirmed the importance of this factor in erythroid development (11, 14), although its role in other

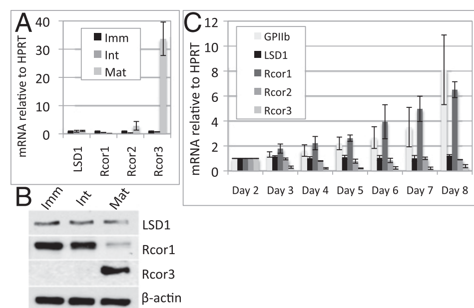

Fig. 7. Expression profiling of LSD1 and Rcor1-3 during erythroid and megakaryocytic maturation. (*A*) qPCR analysis of FACS-sorted immature (Imm), intermediate (Int), and mature (Mat) fetal liver cells (as shown in Fig. 3*A*) for LSD1 and Rcor1-3 mRNA levels, normalized for hypoxanthine phosphoribosyl transferase. (*B*) Western blots of LSD1, Rcor1, and Rcor3 protein expression in the same populations; 25 μg of total protein was loaded in each lane. (*C*) Dynamics of LSD1, Rcor1-3, and GPIIb (glycoprotein IIb; CD41) mRNA expression in in vitro differentiated megakaryocytes.

hematopoietic cells particularly megakaryocytes has not been reported. The tissue restricted phenotype of the Rcor1 deletion relative to that of LSD1 likely indicates compensation from other Rcor proteins chiefly Rcor2 in other tissues. Since Rcor2 is expressed in and required for maintaining pluripotency of ES cells (20), it likely works similarly in other tissues to facilitate LSD1 demethylation. Despite the coexpression, and association of LSD1 and Rcor1 in diverse contexts and with multiple transcription factors (eg, REST, ZEB, Snail) resulting in corecruitment to multiple loci (13, 19, 24, 25–27), the germline Rcor1 deletion produced a surprisingly tissue restricted (erythroid) phenotype. Although the expression of Rcor2 has not been specifically assessed in these cells, it, or other as yet unidentified positve Rcor factors may compensate for the loss of Rcor1 in these cells. While the incomplete expression profile of Rcor2 makes it difficult to predict the extent and severity of its genetic deletion phenotype, the Rcor1 and Rcor2 combined deletions should largely phenocopy that of LSD1 in the absence of other positively acting Rcor factors. Since very little is known about Rcor3 expression or function other than its expression in adult hepatic cells (28), predicting its deletion phenotype is practically impossible. However, our results predict antagonistic phenotypes for Rcor 1/2 versus Rcor3 in their respective regions of expression. Thus, the range and robustness of LSD1 and Rcor1-3 expression should potentially regulate development of multiple cells and tissues, such as ES cells, neuronal cells, endocrine (pituitary) organs (24, 25, 26), and others during mammalian development.

Third, our results have important implications in oncogenesis, where LSD1 is known to repress the expression of tumor suppressors (29) and inhibit normal differentiation programs (16) in cancer stem cells and progenitors. Dampening of LSD1 activity by altering Rcor1-3 levels could potentially reduce the oncogenic impact of this errant demethylase. For instance, LSD1/Rcor1 are recruited by another SNAG domain transcription factor, Snail 1 (17), a potent mediator of epithelial-to-mesenchymal transitions and leukemic transformations, to its gene targets (17, 19, 29). Thus, Rcor3 overexpression in appropriate cells could potentially replace or reduce active Snail/LSD1/Rcor1/2 demethylase complexes with inactive Snail/LSD1/Rcor3 complexes, resulting in reactivation of genes responsible for promoting differentiation, curbing proliferation, or limiting epithelial-to-mesenchymal transitions. Although the mechanisms underlying the apparent anomalies in LSD1 function between normal hematopoiesis, where it promotes differentiation (8, 12, 13, and this study), and during leukemogenesis, where it appears to inhibit differentiation (16, 29), is unclear, identification of LSD1-Rcor targets in different contexts likely would help to resolve this issue. Nonetheless, regardless of the specific repertoire of genes regulated by LSD1 in any given milieu, its H3K4 demethylase activity should be dependent on relative Rcor1-3 stoichiometries and be restricted by Rcor3 overexpression.

Finally, our results potentially could be used to devise alternative strategies for specifically restraining ectopic LSD1 activity with minimal side effects. Because the specificity of Rcor3 inhibition of LSD1 lies in the interaction of the "linker" domain of the former with the "tower" domain of the latter, any protein lacking this "tower" domain [i.e., LSD2 (30) and other amine oxidases] should not be able to interact with Rcor3 (or other Rcors) or be inhibited (or activated) by it/them. Thus, this LSD1-specific effect of the Rcors also should restrict the impact of the natural LSD1 inhibitor, Rcor3, to this demethylase alone.

Materials and Methods

Vectors, Transfection, Immunoprecipitation, and Gel Filtration. V5 epitope-tagged plasmids for Gfi1, Gfi1b, P2A-Gfi1b, and BirA have been described previously (8). Murine Rcor1 (National Center for Biotechnology Information accession no. BC042731), Rcor2 (no. BC055719), and Rcor3 (no. NM_144814) were PCR-amplified from total RNA and cloned into pEF4/myc-His vector (Life Technologies). FLAG-LSD1 was a gift from M. Lee and R. Shiekhattar (2). HEK-293T cells were transiently cotransfected with tagged plasmids in different combinations and harvested after 48 h as described previously (8). Immunoprecipitation was performed with anti-myc antibody and protein G

Sepharose beads (Life Technologies), resolved on SDS/PAGE, and subjected to Western blot analysis as shown. MEL cells were cultured and differentiated as described previously (8, 31). For gel filtration experiments, MEL whole-cell lysates were filtered and loaded onto a Superose 6 10/300 GL column (GE Healthcare) preequilibrated with gel fitration buffer [20 mM Tris·HCl pH 7.5, 100 mM NaCl, 1 mM DTT, and 5% (vol/vol) glycerol]. Fractions (0.3–0.5 mL) were collected and immunoblotted as shown.

Production of Recombinant Proteins. Murine LSD1 (accession no. NM_133872.2) and Rcor1-3 were amplified from total RNA or other vectors and subcloned into pQE TriSystem His.Strep2 (Qiagen) (LSD1), pRSET-B (Life Technologies) (Rcor1), or pRSET-B vectors with engineered C-terminal strep tags (Rcor2/3). LSD1 was expressed in M15 (pREP4), and Rcor1-3 was expressed in BL21DE3. Chimeric Rcor3-SANT1 protein was produced by PCR amplification of the SANT1 domain of Rcor1 (amino acids 87–137) and insertion into the AflII site (amino acid 268) of Rcor3pRSETB. Rcor3-SANT2 protein was produced by amplification of the SANT2 domain of Rcor1 (amino acids 260–382) and insertion into the AflII–HindIII sites of Rcor3pRSETB, replacing amino acids 268–395 of Rcor3. Recombinant proteins were induced with 1 mM IPTG at 25 °C for 5–7 h, purified using Talon metal affinity (Clontech) and Strep-Tactin (Qiagen) resins, and then eluted with 150 mM imidazole and 2.5 mM desthiobiotin, respectively. Purified proteins were dialyzed, quantified by the Bradford method, and visualized by Coomassie blue-stained SDS/PAGE.

Mononucleosome Preparation and Demethylation Assays. Mononucleosomes were prepared as described previously (32). In brief, 2.5×10^8 HeLa cells were pelleted and homogenized to obtain oligonucleosomes, digested with micrococcal nuclease, and subjected to 10–40% glycerol gradient sedimentation. Histone and nucleosomal demethylation assays were performed as described previously (3). In brief, bulk histones or mononucleosomes were incubated with purified proteins as indicated in the histone demethylase assay buffer (50 mM Tris pH 8.5, 50 mM KCl, 5 mM MgCl, 1 mM DTT, and 5% glycerol) at 37 °C for 4 h, and the reaction mix was analyzed by Western blotting with anti-dimethyl H3K4 (Millipore; 07-030), anti-dimethyl H3K9 (Abcam; 1220-100), and anti-histone H3 (Upstate Biologicals; 05-499) antibodies.

Lentivirus Production and Primary Cell Manipulations. ShRNAs were either obtained from the Mission collection (Rcor1-3) (Sigma-Aldrich) or generated (LSD1 shRNAs) by subcloning shRNA sequences into pLKO.1 (Addgene). Viral expression vectors were prepared by subcloning Rcor1-3 cDNAs into pCDH-MSCV (Systems Biosciences). Lentiviral particles were produced in HEK-293T cells by cotransfecting shRNA/expression plasmids with the pPAX2 packaging and pMD2.G envelope plasmids. Viral supernatants were collected after 48 h and filtered before use. Then 10^5 fetal liver cells were harvested from day 12.5 embryos (E12.5) and infected with lentiviral supernatants in 8 μg/mL of polybrene. Transduced cells were cultured in IMDM media supplemented with erythroid cytokines (erythropoietin, 2 U/mL; stem cell factor, 25 ng/mL) or megakaryocytic cytokines (thrombopoietin, 20 ng/mL; IL-3, 10 ng/mL) and puromycin (1 μg/mL) and harvested after 4–8 d.

Histological Staining, Quantitative RT-PCR, and Flow Cytometry Analyses. Cells were cytocentrifuged onto slides and subjected to benzidine and acetylcholine esterase staining as described previously (21). Numbers of positively staining cells relative to total number of cells were determined using ImageJ cell imaging and counting software (33). Total RNA was isolated and subjected to quantitative RT-PCR (qRT-PCR) analysis using primers as described previously (8) and listed in *SI Materials and Methods*, and expression was normalized to that for hypoxanthine phosphoribosyl transferase. For FACS analysis, $>10^5$ cells were stained with anti-mouse CD9-FITC, anti–c-kit-PE, and anti-mouse CD41-APC or ter119-APC antibodies (eBioscience), as indicated. Sorting and analyses were performed on a BD FACSAria cell sorter and a BD LSRII analyzer, respectively.

ChIP. ChIP was performed as described previously (8, 31) with anti-Gfi1b (Santa Cruz Biotechnology; sc-8559X), anti-LSD1 (Abcam; ab17721), anti-Rcor3 (custom), and anti–di-meH3-K4 (Millipore; 07030) antibodies. Polyclonal anti-mouse rabbit serum was produced against the C-terminal 17 amino acids of Rcor3, affinity-purified, and used for Western blot analyses and ChIP. ChIP primers used were as described previously (8, 31) and listed in *SI Materials and Methods*.

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1. Shi Y, et al. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119(7):941–953.
2. Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. *Nature* 437(7057):432–435.
3. Shi YJ, et al. (2005) Regulation of LSD1 histone demethylase activity by its associated factors. *Mol Cell* 19(6):857–864.
4. Metzger E, et al. (2005) LSD1 demethylates repressive histone marks to promote androgen receptor-dependent transcription. *Nature* 437(7057):436–439.
5. Garcia-Bassets I, et al. (2007) Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* 128(3):505–518.
6. Yang M, et al. (2006) Structural basis for CoREST-dependent demethylation of nucleosomes by the human LSD1 histone demethylase. *Mol Cell* 23(3):377–387.
7. Baron R, Vellore NA (2012) LSD1/CoREST is an allosteric nanoscale clamp regulated by H3-histone-tail molecular recognition. *Proc Natl Acad Sci USA* 109(31):12509–12514.
8. Saleque S, Kim J, Rooke HM, Orkin SH (2007) Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. *Mol Cell* 27(4):562–572.
9. Hu X, et al. (2009) LSD1-mediated epigenetic modification is required for TAL1 function and hematopoiesis. *Proc Natl Acad Sci USA* 106(25):10141–10146.
10. Li Y, et al. (2012) Dynamic interaction between TAL1 oncoprotein and LSD1 regulates TAL1 function in hematopoiesis and leukemogenesis. *Oncogene* 31(48):5007–5018.
11. Xu J, et al. (2013) Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A. *Proc Natl Acad Sci USA* 110(16):6518–6523.
12. Sprüssel A, et al. (2012) Lysine-specific demethylase 1 restricts hematopoietic progenitor proliferation and is essential for terminal differentiation. *Leukemia* 26(9):2039–2051.
13. Kerenyi MA, et al. (2013) Histone demethylase Lsd1 represses hematopoietic stem and progenitor cell signatures during blood cell maturation. *Elife* 2:e00633.
14. Yao H, et al. (2014) The co-repressor Rcor1 is essential for murine erythropoiesis. *Blood*, 10.1182/blood-2013-11-538678.
15. Harris WJ, et al. (2012) The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. *Cancer Cell* 21(4):473–487.
16. Schenk T, et al. (2012) Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. *Nat Med* 18(4):605–611.
17. Lin Y, et al. (2010) The SNAG domain of Snail1 functions as a molecular hook for recruiting lysine-specific demethylase 1. *EMBO J* 29(11):1803–1816.
18. Laurent B, et al. (2012) A short Gfi-1B isoform controls erythroid differentiation by recruiting the LSD1-CoREST complex through the dimethylation of its SNAG domain. *J Cell Sci* 125(Pt 4):993–1002.
19. Ferrari-Amorotti G, et al. (2013) Inhibiting interactions of lysine demethylase LSD1 with snail/slug blocks cancer cell invasion. *Cancer Res* 73(1):235–245.
20. Yang P, et al. (2011) RCOR2 is a subunit of the LSD1 complex that regulates ESC property and substitutes for SOX2 in reprogramming somatic cells to pluripotency. *Stem Cells* 29(5):791–801.
21. Saleque S, Cameron S, Orkin SH (2002) The zinc-finger proto-oncogene Gfi-1b is essential for development of the erythroid and megakaryocytic lineages. *Genes Dev* 16(3):301–306.
22. Zhang J, Socolovsky M, Gross AW, Lodish HF (2003) Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: Functional analysis by a flow cytometry-based novel culture system. *Blood* 102(12):3938–3946.
23. Liu L, et al. (2013) Histone lysine-specific demethylase 1 (LSD1) protein is involved in Sal-like protein 4 (SALL4)-mediated transcriptional repression in hematopoietic stem cells. *J Biol Chem* 288(48):34719–34728.
24. Wang J, et al. (2007) Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature* 446(7138):882–887.
25. Lunyak VV, et al. (2002) Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* 298(5599):1747–1752.
26. Shi Y, et al. (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 422(6933):735–738.
27. Lin T, Ponn A, Hu X, Law BK, Lu J (2010) Requirement of the histone demethylase LSD1 in Snai1-mediated transcriptional repression during epithelial-mesenchymal transition. *Oncogene* 29(35):4896–4904.
28. Xue JH, et al. (2011) Involvement of REST corepressor 3 in prognosis of human hepatitis B. *Acta Pharmacol Sin* 32(8):1019–1024.
29. Murray-Stewart T, Woster PM, Casero RA, Jr. (2014) The re-expression of the epigenetically silenced e-cadherin gene by a polyamine analogue lysine-specific demethylase-1 (LSD1) inhibitor in human acute myeloid leukemia cell lines. *Amino Acids* 46(3):585–594.
30. Karytinos A, et al. (2009) A novel mammalian flavin-dependent histone demethylase. *J Biol Chem* 284(26):17775–17782.
31. Chowdhury AH, et al. (2013) Differential transcriptional regulation of meis1 by Gfi1b and its co-factors LSD1 and CoREST. *PLoS ONE* 8(1):e53666.
32. Utley RT, et al. (1996) In vitro analysis of transcription factor binding to nucleosomes and nucleosome disruption/displacement. *Methods Enzymol* 274:276–291.
33. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9(7):671–675.
34. le Maire M, et al. (2008) Gel chromatography and analytical ultracentrifugation to determine the extent of detergent binding and aggregation, and Stokes radius of membrane proteins using sarcoplasmic reticulum $Ca2^+$-ATPase as an example. *Nat Protoc* 3(11):1782–1795.