Deconstructing repression: evolving models of co-repressor action

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Abstract | A crucial aspect of development, homeostasis and prevention of disease is the strict maintenance of patterns of gene repression. Gene repression is largely achieved by the combinatorial action of various enzymatic complexes — known as co-repressor complexes — that are recruited to DNA by transcription factors and often act through enzymatic modification of histone protein tails. Our understanding of how co-repressors act has begun to change over recent years owing to the increased availability of genome-scale data. Here, we consider specific strategies that underlie repression events — for example, those mediated by the nuclear receptor co-repressor (NCoR, also known as NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT, also known as NCOR2) co-repressor complexes — and discuss emerging themes in gene repression.

From the first cell division to the complex integration of signalling pathways in differentiated tissues, the multicellular organism must precisely regulate transcription to ensure correct gene expression. Gene activation and repression are mostly regulated through changes in chromatin structure imparted by DNA methylation, chromatin remodelling and histone modifications. The proteins required to accomplish chromatin regulation are many and varied, and include DNA methyltransferases, chromatin-remodelling complexes, DNA-binding transcription factors and chromatin-modifying complexes. Chromatin-remodelling complexes use ATP hydrolysis to modify nucleosome structure and modulate the accessibility of DNA for transcription factors (for a review, see REF. 1). Co-activators and co-repressors have a major role in altering chromatin structure through the modification of core histone amino-terminal tails. These modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation (for reviews, see REFS 2–4). The combination of histone modifications has been suggested to constitute a 'histone code' that directs specific and distinct transcriptional programmes[5].

The mechanisms by which co-activator complexes drive activation events and the chromatin modifications that influence gene activation have been characterized in some detail. Modifications associated with repression (repressive marks) — including histone deacetylation and specific cases of methylation and ubiquitylation — are thought to act together to impose a higher order structure on chromatin that creates an environment that is not permissive to gene transcription. However, the mode of action and specificity of co-repressors and the histone modifications they mediate are much less well understood than for co-activators.

Recently, developments in the techniques used to study gene regulation on a genome-wide scale have allowed a more integrated view of the dynamics of chromatin modifications and have increased our understanding of co-repressor actions. Genome-scale data have resulted in a reassessment of the original model of co-repressor activity, which assumed that co-repressors associate with repressed genes and are replaced by co-activators during gene activation[6]. In this Review, we discuss the emerging concept that long-term gene repression is probably maintained not by the constitutive presence of co-repressor complexes but by histone modifications that are maintained by intermittent co-repressor activity. Current models of co-repressor function appreciate the dynamics of the opposing co-activator and co-repressor complexes, which seem to continually cycle on and off DNA. As genome-wide data continue to accrue, co-repressor complexes may turn out to be as important in gene-activation events as in repression owing to, for example, their ability to reset chromatin for subsequent rounds of transcription.

In this Review, we focus mainly on the examples of histone deacetylation and the histone deacetylase (HDAC)-containing nuclear receptor co-repressor (NCoR, also known as NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT, also known as NCOR2) co-repressor complexes[7–11].

Methylation
The enzymatic process of adding a methyl group to a lysine or an arginine residue on histone tails or other proteins. Alternatively, methyl groups can be added to DNA itself on cytosine bases.

Histone
A family of small, highly conserved basic proteins that are found in the chromatin of all eukaryotic cells and that associate with DNA to form a nucleosome. Two each of the core histones H2A, H2B, H3 and H4 make up an octameric nucleosome, around which DNA winds.

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These have been relatively well studied compared with other co-repressor complexes, and we use them to discuss general mechanisms of co-repressor recruitment and activity and the specific regulation of their functions. We also consider the roles of co-repressors in development and disease. Although we focus on the role of these and other co-repressor complexes in mammalian systems — including discussion of *in vivo* mouse models of co-repressor function — much of the pioneering work on enzymatic modification of histones has been done in yeast, and many co-repressors have been well characterized in *Drosophila melanogaster*. We refer readers to recent reviews concerning models of co-repression in different species[4,12–14]. Interestingly, specific co-repressors might have evolved recently. For example, yeast and *D. melanogaster* have analogues of NCoR and SMRT, but not true homologues[15,16]. There might, however, be conserved principles of repression across species.

Co-repressor complexes and enzymatic activities

Enzymatic modifications of histone tails that mediate repression include arginine deimination, changes in the methylation status of particular lysine residues, histone lysine deacetylation, and histone ubiquitylation (FIG. 1). These modifications are imposed by various enzymes that are recruited to DNA directly by transcription factors or indirectly by other co-repressors or histone modifications. Intriguingly, recent work has also shown that non-coding RNAs can be instrumental for the recruitment of chromatin-modifying repressor complexes[17]. In this section, we introduce the complexes that mediate gene repression and their enzymatic activities. We do not discuss each complex in detail but rather comment on the general concept that modifications work in concert rather than in isolation. In FIG. 1, we have attempted to illustrate the complexity and integration of co-repressor action. TABLE 1 lists, as comprehensively as possible considering space limitations, known co-repressors and their associated enzymatic activities or functions, in addition to references for further information. The examples of HDAC activity and the NCoR and SMRT co-repressor complexes are discussed in more detail by way of illustration.

For transcriptional activation, it has become clear that distinct modifications act together during gene regulation and that certain modifications can be responsible for the recruitment of specific classes of co-activator complex. However, how modifications that lead to gene repression act in combination and which specific modifications recruit specific co-repressor complexes have not been investigated as thoroughly[3]. It is well appreciated, however, that many co-repressor complexes combine distinct enzymatic activities. For example, HDAC activity is often coupled with other chromatin-binding or chromatin-remodelling activities (FIG. 1). The nucleosome remodelling and histone deacetylation (NURD) complex is particularly interesting, as it combines several complementary enzymatic functions in a single large complex. The NURD complex was initially found to include HDACs and chromatin-remodelling ATPases, and now lysine-specific histone demethylase 1 (LSD1) has also been identified as a component[18]. This suggests that important aspects of epigenetic regulation, such as the crosstalk between histone deacetylation and demethylation, can be regulated by intrinsic components of a single regulatory complex.

Although histone acetylation and deacetylation are clearly associated with transcriptional activation and repression, respectively, in general the type of modification does not predict whether the transcriptional outcome will be positive or negative. For example, histone ubiquitylation is generally associated with transcriptional activation, but ubiquitylation of histone H2A has been shown to mediate transcriptional repression. Histone H2B ubiquitylation is associated with transcriptional activation and is required for methylation of histone H3 at lysine 4 and lysine 79, therefore providing a good example of the interdependency of different histone modifications (for reviews, see REFS 19,20).

Histone deacetylases. The HDACs are a large protein family, as might be expected when one considers that histone deacetylation is one of the most important processes that mediate transcriptional repression. In vertebrates, the family of 'classic' HDACs contains 11 members (HDAC1–HDAC11). These can be divided into three subclasses: class I (HDAC1–HDAC3 and HDAC8), class II (HDAC4–HDAC7, HDAC9 and HDAC10) and class IV (HDAC11). Class III deacetylases are NAD^+-dependent deacetylases of the sirtuin family (for dedicated reviews, see REFS 21–24).

In general, acetylation is associated with relaxation of chromatin structure and therefore with increased transcriptional activity; removal of acetyl groups from histone tails induces a condensation in DNA structure that prevents gene transcription. Indeed, histone tails at repressed gene loci are globally hypoacetylated, whereas hyperacetylated chromatin is transcriptionally active. However, deacetylation does not occur independently from other modifications. Several different co-repressor complexes, each of which contains complementary factors that carry additional enzymatic activities, mediate the recruitment of HDACs to chromatin, therefore allowing coordinated epigenetic modifications, as discussed above.

Class I deacetylases are catalytic subunits of various multiprotein complexes that are responsible for transcriptional repression and chromatin remodelling. HDAC1 and HDAC2, for example, have been identified in a number of co-repressor complexes, including the SIN3A co-repressor complex[25,26], the co-repressor for RE1 silencing transcription factor (CoREST, also known as neural-restrictive silencing factor and RCOR1) complex[27–29], and the NURD complex[30–33]. HDAC3 is recruited to promoters by association with the NCoR or SMRT co-repressors.

NCoR and SMRT. The NCoR and SMRT co-repressors show high homology at the protein-sequence level, and their complexes have similar composition. In addition to HDAC3, the core complexes contain the proteins transducin β-like 1 (TBL1, also known as TBL1X),

Acetylation
The enzymatic process of adding an acetyl group to a lysine residue on histone tails or on other proteins.

Ubiquitylation
The enzymatic process of covalently conjugating a protein with single copies or chains of ubiquitin. Ubiquitin is an 8.5-kDa protein that exists in all eukaryotic cells.

Sumoylation
The enzymatic process of covalently conjugating a protein with the small protein SUMO.

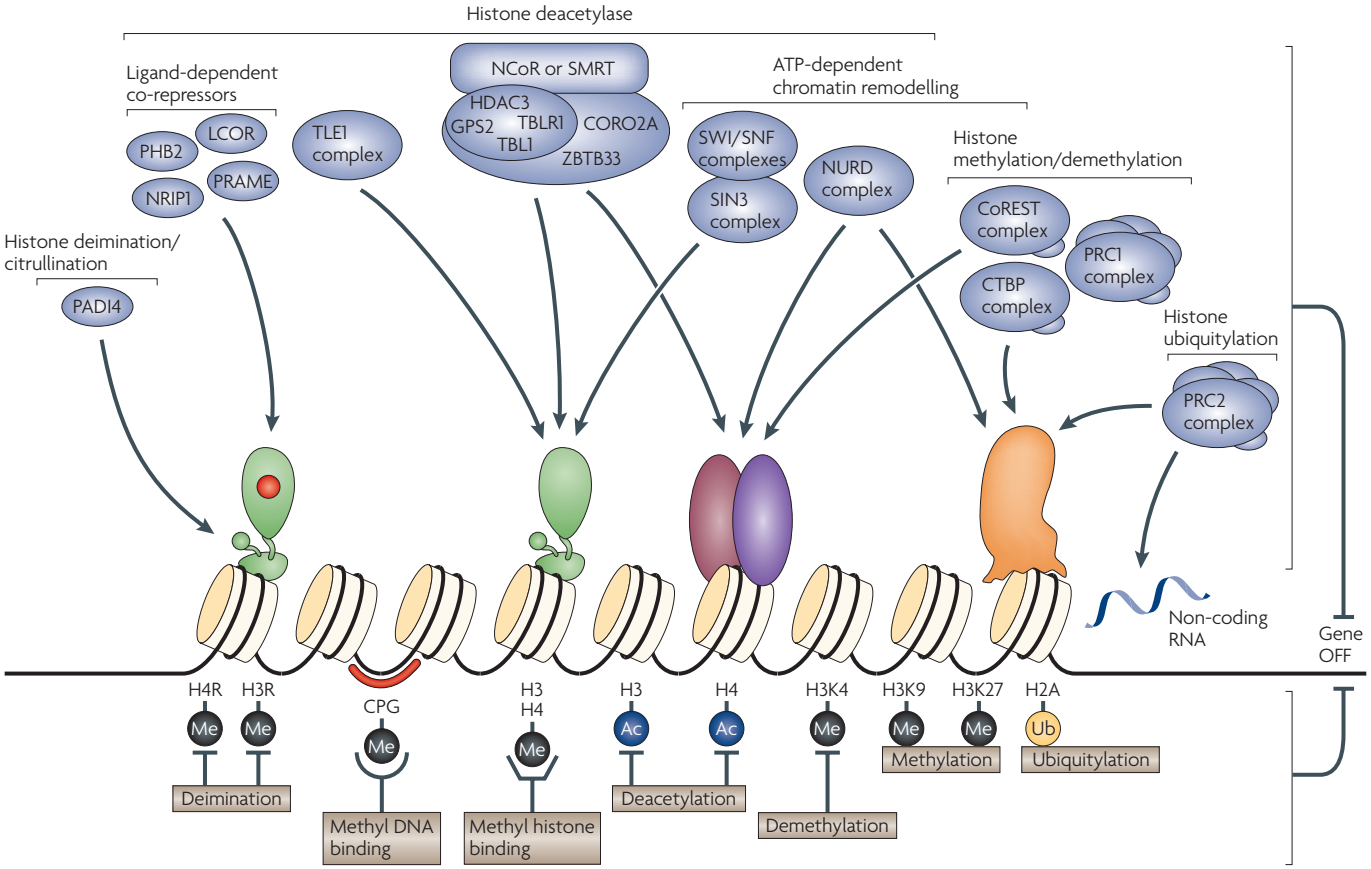

Figure 1 | **Co-repressor complexes and associated enzymatic activities.** A putative repressed transcription unit is represented, with various co-repressor complexes (grey). The co-repressors can be recruited by different transcription factors (green shapes with or without a red circle, which represents a ligand), including dimeric transcription factors (brown and purple ovals) and monomeric transcription factors (orange shape). The bars at the top of the figure group together complexes that carry similar enzymatic functions. At the bottom, below the nucleosomal DNA, are the histone tail modifications that are mediated by the above complexes. Also shown at the bottom of the figure is the recruitment of these complexes by specific modifications, such as DNA methylation (for example, at a methylated CpG island (red curve)) or histone tail methylation, or by non-coding RNA. CoREST, co-repressor for RE1 silencing transcription factor (also known as neural-restrictive silencing factor and RCOR1); CORO2A, coronin 2A (also known as IR10); CTBP, carboxy-terminal-binding protein; GPS2, G-protein-pathway suppressor 2; HDAC3, histone deacetylase 3; LCOR, ligand-dependent co-repressor; NCoR, nuclear receptor co-repressor (also known as NCOR1); NRIP1, nuclear receptor-interacting protein 1 (also known as RIP140); NURD, nucleosome remodelling and histone deacetylation; PADI4, peptidyl arginine deiminase type 4; PHB2, prohibitin 2; PRAME, preferentially expressed antigen in melanoma; PRC, polycomb repressive complex; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor (also known as NCOR2); SWI/SNF, switch/sucrose non-fermentable; TBL1, transducin β-like 1 (also known as TBL1X); TBLR1, transducin β-like-related 1 (also known as TBL1XR1); TLE1, transducin-like enhancer of split 1; ZBTB33, zinc finger and BTB domain-containing 33 (also known as Kaiso).

TBL-related 1 (TBLR1, also known as TBL1XR1) and G-protein-pathway suppressor 2 (GPS2)[34–36]. Other proteins that have been shown to bind to the NCoR and SMRT complexes include TAK1-binding protein 2 (TAB2, also known as MAP3K7IP2)[37], coronin 2A (CORO2A, also known as IR10)[36] and zinc finger and BTB domain-containing 33 (ZBTB33, also known as Kaiso)[38]. Interestingly, ZBTB33 seems to discriminate between NCoR and SMRT, as it was shown to bind exclusively to the NCoR complex[38]. Neither NCoR nor SMRT has been found to stably associate with any of the other co-repressor complexes or enzymatic subunits other than HDAC3. Therefore, HDAC3 is likely to be the primary enzyme responsible for the deacetylase activity that is associated with NCoR- and SMRT-mediated repressive events. However, other HDACs or HDAC-containing complexes can be recruited in a transcription factor-specific or context-specific manner by less stable interactions with NCoR and SMRT[39–41].

Evolving models of co-repressor function

Given the complex subunit composition of co-repressor complexes, can a unifying model be formed of how co-repressors act in gene regulation? Models of co-repressor function have been proposed since these

Nuclear hormone receptors
A large family of DNA-binding transcription factors that are responsible for sensing various hormonal and environmental stimuli and mediating gene expression accordingly.

complexes were first identified, but recent years have seen a conceptual shift owing to the increased availability of genome-scale data on protein–DNA interactions, chromatin modifications and gene expression. Here, we discuss models of co-repressor action and how they are changing.

The classic model: recruitment by transcription factors. Over a decade ago, the first co-repressor proteins were identified as interacting partners of unliganded nuclear hormone receptors, and co-activators were found to bind specifically to liganded receptors. This led to the proposal of a simple two-step model of transcriptional regulation

Table 1 | **The components of co-repressor complexes and their associated enzymatic activities**

Co-repressor	Complex	Enzymatic activity/function	Refs
BAF proteins	SWI/SNF	DNA and histone binding	129,130
BMI1	PRC1	H2A ubiquitylation cofactor	131
BRG1	SWI/SNF	ATPase	129,130
CBX4	PRC1	H3K27 trimethylation	131,132
CHD4	NURD	ATPase	133
CoREST	CoREST	*	134
CORO2A	NCoR, SMRT	Actin binding	‡
CTBP1, CTBP2	CoREST	NAD-dependent dehydrogenase	134,135
EED, EZH2	PRC2	H3K27 methylation	131,132,136
GPS2	NCoR, SMRT	Kinase inhibition	‡
HDAC1, HDAC2	SIN3, NURD, CoREST	Deacetylation	24,133,137
HDAC3	NCoR, SMRT	Deacetylation	24
HDAC4–HDAC11	§	Deacetylation	22,24
KAP1	NCoR, SMRT	Histone binding/scaffold	138
LSD1	CoREST, NURD	H3K4 demethylation	134,139
MBD3	NURD	Methyl DNA binding	133,140
MTA1–MTA3	NURD	*	133
NCoR, SMRT	NCoR, SMRT	*	‡
PARP1	TLE1, CTCF	Poly-ADP ribosylation	141
PHC1, PHC2	PRC1	*	131,132
RBAP46, RBAP48	SIN3	H4 binding	142
RING1	PRC1	H2AK119 ubiquitylation	131,132
SAP18	SIN3	*	142
SAP30	SIN3	DNA binding/bending	142
SIN3	SIN3, CoREST	*	137,142
SIRT1–SIRT5	§	NAD-dependent deacetylation	21,143
SUZ12	PRC2	H3K27 methylation stimulation	131,132,136
TAB2	NCoR, SMRT	Ubiquitin binding/shuttling	‡
TBL1, TBLR1	NCoR, SMRT	Ubiquitylation	‡
TLE1–TLE5	TLE	Histone binding/oligomerization	12
ZBTB33	NCoR	Methyl DNA binding	140

*No specific enzymatic function has been described for the corresponding protein. ‡This protein is discussed in this Review. §This protein has not been found to be associated with any of the major co-repressor complexes. BAF, BRG1-associated factor; BRG1, BRM/SWI2-related gene 1 (also known as SMARCA4); CBX4, chromobox homologue 4 (also known as PC2); CHD4, chromodomain helicase DNA-binding protein 4; CoREST, co-repressor for RE1 silencing transcription factor (also known as neural-restrictive silencing factor and RCOR1); CORO2A, coronin 2A (also known as IR10); CTBP, carboxy-terminal-binding protein; CTCF, CCCTC-binding factor complex; EED, embryonic ectoderm development; EZH2, enhancer of zeste 2; GPS2, G-protein-pathway suppressor 2; HDAC, histone deacetylase; KAP1, KRAB-associated protein 1 (also known as TIF1β and TRIM28); LSD1, lysine-specific histone demethylase 1; MBD3, methyl-CpG-binding domain protein 3; MTA, metastasis-associated; NCoR, nuclear receptor co-repressor (also known as NCOR1); NURD, nucleosome remodelling and histone deacetylation; PARP1, poly(ADP-ribose) polymerase 1; PHC, polyhomeotic-like; PRC, polycomb repressive complex; RBAP46, retinoblastoma-binding protein 46 (also known as RBPP7); RBAP48, retinoblastoma-binding protein 48 (also known as RBBP4); RING1, ring finger protein 1; SAP, SIN3-associated polypeptide; SIRT, sirtuin; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor (also known as NCOR2); SUZ12, suppressor of zeste 12; SWI/SNF, switch/sucrose non-fermentable; TAB2, TAK1-binding protein 2 (also known as MAP3K7IP2); TBL1, transducin β-like 1 (also known as TBL1X); TBLR1, transducin β-like-related 1 (also known as TBL1XR1); TLE, transducin-like enhancer of split; ZBTB33, zinc finger and BTB domain-containing 33 (also known as Kaiso).

in which an activating signal — ligand binding, in the case of nuclear hormone receptors — is the key event that causes a unidirectional switch between co-repressors and co-activators (FIG. 2A, reviewed in REF. 42).

Numerous biochemical studies have dissected in detail the mechanisms of interaction between nuclear hormone receptors and various co-regulatory proteins. This has resulted in a simple, elegant model in which the unliganded receptor has an open conformation that allows interaction with co-repressors. A motif called the LxxH/IIxxxI/L or co-repressor nuclear receptor (CoRNR) box motif in the co-repressor interacts with a hydrophobic groove on the receptor[43,44]. Upon agonist binding, a conformational change in the receptor causes loss of binding of the co-repressor and replacement by a co-activator. The co-activator interacts with the receptor through an LxxLL binding motif, also called the nuclear receptor box. Binding of co-repressors and binding of co-activators to nuclear receptors were found to be mutually exclusive because both occur at the same interaction surface on the receptor. Ligand-induced repositioning of helix 12 of the receptor reduces the amplitude of the hydrophobic groove so that the shorter LxxLL fits but the CoRNR box does not[45]. Therefore, this original view of an exchange between co-activator or co-repressor complexes (reviewed in REF. 42) considered the presence of a co-activator versus a co-repressor complex as the key 'switch' between transcriptional activation or repression; histone acetyltransferases (HATs) are associated with active genes and HDACs are associated with inactive genes (FIG. 2A).

Cyclical model. This simple model was first refined when Gannon and colleagues[46] demonstrated that induction of transcription of trefoil factor 1 (*TFF1*) by liganded oestrogen receptor in a human cell line required cyclical recruitment of HDACs and nucleosome-remodelling complexes in addition to co-activators. They proposed a cyclical model in which DNA-binding factors and their associated cofactors are continuously turned over on responsive promoters: a continuous exchange between co-repressor and co-activator complexes — with dismissal of the co-r epressors at the beginning of each new cycle — is required for transcriptional activation (FIG. 2B). Indeed, the presence of, or even requirement for, co-repressors on several promoters during activation events led researchers to re-examine promoter occupancy by various cofactors. Similar cyclical models have now been proposed for nuclear factor-κB (NF-κB)-dependent, Wnt-regulated and peroxisome proliferator-activated receptor-γ (PPARγ)-specific target genes[47–49].

Implicit in the cyclical model is the idea that transition from gene repression to gene activation *in vivo* requires both a conformational change in the receptor and active release of the co-repressors in response to ligand binding. There is evidence that diverse signalling pathways converge on the co-repressor complexes and mediate the removal of the co-repressor-associated HDAC activities. This active release of co-repressors as a requirement for gene activation is generally referred to as 'de-repression' (see further discussion below).

Combinatorial model of co-repressor and co-activator action. Recently, new evidence has emerged that suggests further development of our model of co-repression is needed. Although in-depth promoter-specific studies are crucial to fully understand how different transcription factors and their associated machinery cooperate to regulate each gene in a context-specific fashion, genome-wide mapping of transcription factors and other regulatory factors has proven to be indispensable for understanding how the regulation of transcription occurs on a large scale. The combination of chromatin immunoprecipitation (ChIP) analysis with microarrays and, more recently, with next-generation DNA sequencing, has enabled genome-scale analyses of protein–DNA interactions and histone modifications (for reviews, see REFS 50,51). Such studies have included the profiling of several histone marks and transcription factors in different cell models[50,52,53].

Genome-wide profiling of transcriptional cofactors has recently allowed a global view of how co-activator and co-repressor proteins associate with the genome. TATA box-binding protein (TBP)-associated factor 1 (TAF1) was found to bind to promoter regions, consistent with its role in initiation of transcription, and the HAT protein p300 was described as a marker for enhancer regions[54,55]. Subsequent work in human and mouse embryonic stem cells localized components of polycomb repressor complexes (PRCs) to genes marked by histone H3 trimethylated at lysine 27 (H3K27me3), which are repressed but primed for activation[56,57]. Interestingly, in human embryonic fibroblasts, PRC proteins are localized to genes that will be repressed during differentiation as well as to repressed genes, which suggests that PRC proteins might mark some genes for subsequent repression[58].

Wang and colleagues[59] recently analysed the genome-wide localization of several HDAC proteins and some of their HAT counterparts in human $CD4^+$ T cells. They found — somewhat surprisingly — that HDAC proteins were not recruited to silenced gene promoters. Instead, both HATs and HDACs were enriched on inactive promoters that seem to be primed for activation — as shown by the presence of H3K4 methylation — and were also enriched on active promoters. The presence of HDACs on primed promoters suggests that deacetylation is important to prevent RNA polymerase II binding to genes that are ready to be activated but should not yet be switched on. For example, the HDACs might remove undesired basal acetylation. Unexpectedly, HDACs were detected on active transcription units in greater abundance than primed promoters. This result suggests a major role for HDACs in the maintenance of gene activation[59] (FIG. 2C). These data are consistent with previous studies of the oestrogen-responsive *TFF1* promoter and the PPARδ-dependent pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) gene — these analyses identified a well-defined periodicity of gene transcription phases with associated alternate recruitment of co-repressor and co-activator complexes[46,60] (FIG. 2B). Indeed, HDACs might be recruited to counteract the histone acetylation carried out by HATs, high levels of

Chromatin immunoprecipitation
A technique that is used to identify potential regulatory sequences by isolating soluble DNA chromatin extracts (complexes of DNA and protein) using antibodies that recognize specific DNA-binding proteins.

Polycomb
A class of proteins — originally described in *Drosophila melanogaster* — that maintain the stable and heritable repression of several genes, including the homeotic genes.

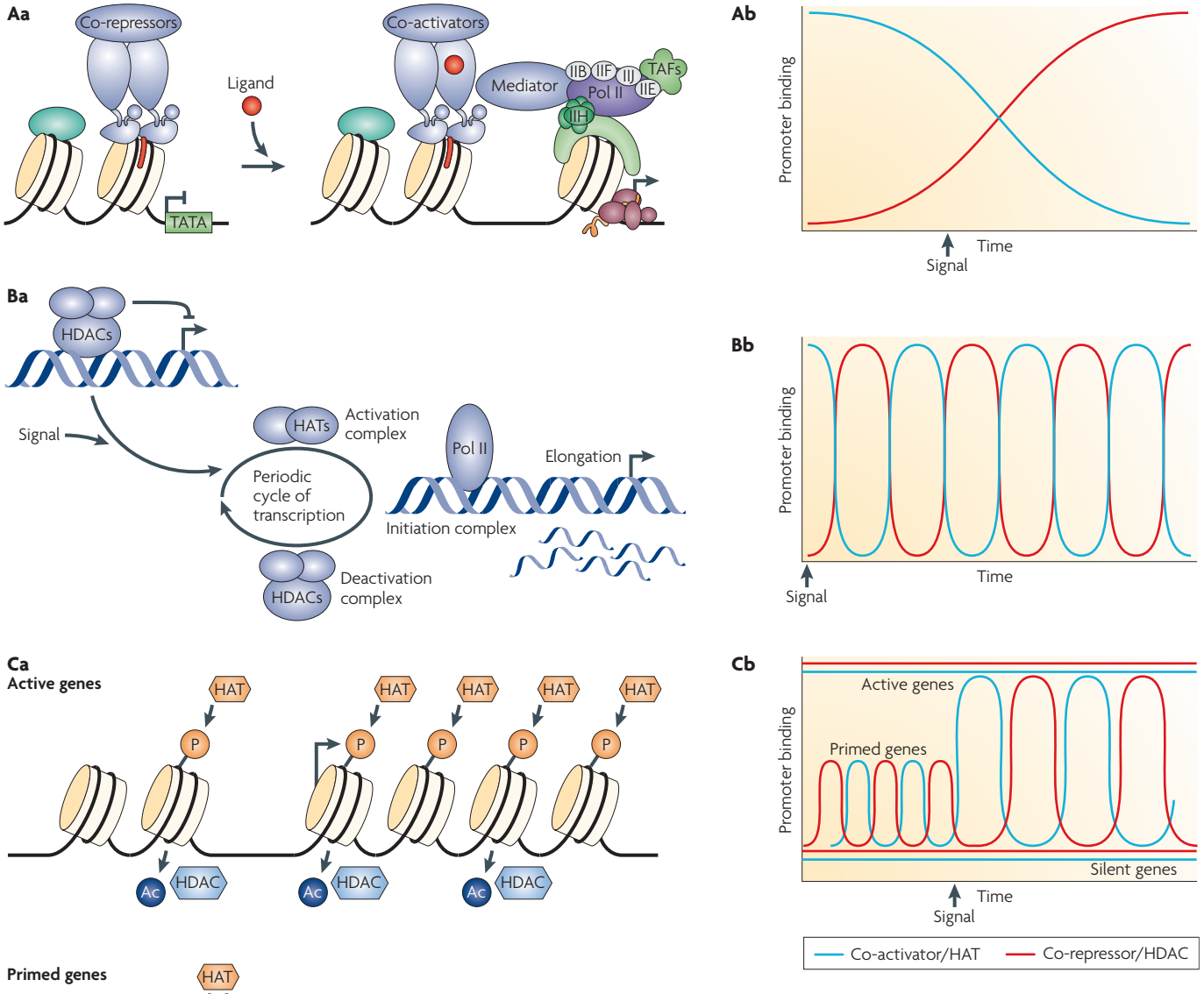

Figure 2 | **Three progressive models of transcriptional regulation by HAT- and HDAC-containing complexes.** **Aa** | The classic model of a signal-dependent switch between co-repressors and co-activators. Co-repressors are recruited by the unliganded nuclear receptor to a repressed promoter. Upon ligand binding they are dismissed and co-activators are recruited to the active transcription unit. **Ab** | The simple exchange between co-repressor and co-activators as measured by promoter occupancy. **Ba** | Ligand-induced regulation of hormone-dependent genes with cyclic recruitment and dismissal of co-repressor and co-activator complexes. **Bb** | In this case, co-repressors and co-activators are continuously exchanged on the promoter. **Ca** | Summary of a recent genome-wide location analysis for histone deacetylases (HDACs) and histone acetyltransferases (HATs). Three classes of regulated genes have been identified: silent, primed and active genes[59]. **Cb** | Co-activators and co-repressors cycle on and off both primed and active genes. Ac, acetylation; K4me3, trimethylation at lysine 4; K27me3, trimethylation at lysine 27; IIB/IIE/IIF/IIH/IIJ, transcription factor IIB/IIE/IIF/IIH/IIJ; P, phosphorylation; PCG, polycomb group complex; Pol II, RNA polymerase II; TAF, TATA box-binding protein (TBP)-associated factor. Part **c** is modified, with permission, from REF. 59 © (2009) Elsevier.

which might destabilize chromatin to a point at which successive rounds of transcription are inhibited. It is proposed that HDACs would then remove acetyl groups, therefore resetting the chromatin structure for the next round of transcription.

It is important to note that the study by Wang and colleagues is only a first snapshot of a very complex and integrated system. The analysis is restricted to one type of histone-modifying co-repressor protein — HDACs. Also, as it was performed in resting T cells, it records a picture for steady-state gene transcription rather than for transcription in response to an activating signal, such as nuclear receptor ligands or inflammatory responses. Therefore, more studies are required to investigate whether the observations made for HDACs will hold true for other co-repressors. Such further studies will enable researchers to obtain a fuller picture of the genome-wide localization of co-repressors during different developmental stages, and thereby understand their cooperative actions in transcriptional regulation and their misregulation in disease.

De-repression

As noted above, a concept that has emerged from studies of co-repression is that there are active mechanisms involved in the exchange of co-repressors for co-activators. Active removal of co-repressors is known as de-repression. De-repression can be achieved by different mechanisms. One mechanism is direct phosphorylation and nuclear export of the co-repressors. For example, inhibitor of NF-κB kinase subunit α (IKKα), mitogen-activated protein kinase kinase kinase 1 (MAP3K1) and AKT can phosophorylate NCoR and SMRT and cause their relocation[37,49,61,62]. Differential responses to some of these kinase signalling cascades are also responsible for providing some level of specificity between NCoR and SMRT (which is discussed below). Alternatively, de-repression can occur through an ubiquitylation-dependent step. In the case of the NCoR and SMRT co-repressor complexes, two specific nuclear co-repressor exchange (NCoEx) factors, TBL1 and TBLR1, were identified as being required for the ubiquitylation-dependent dismissal of the co-repressors[63]. Interestingly, as intrinsic components of the NCoR and SMRT repressor complexes, TBL1 and TBLR1 are also required for the mediation of repression[64,65], which suggests that co-repressors that are localized at a repressed promoter are already primed for release. The signals that promote gene induction must activate parallel pathways, both to activate the transcriptional machinery and to relieve repression imposed by the presence of a co-repressor complex. Although the specific details of how the exchange machinery gets activated in response to ligand induction have not been yet fully characterized, some details of the specificity between TBL1 and TBLR1 and of their ability to dismiss distinct co-repressor proteins have emerged. As TBLR1 was found to be responsible for the clearance of NCoR and SMRT, we speculate that these two co-repressors could be direct targets of TBLR1- and ubiquitin-conjugating enzyme E2 D1 (UBE2D1)-mediated ubiquitylation. By contrast, TBL1 was found to be specific for the dismissal and degradation of the co-repressors carboxy-terminal-binding protein 1 (CTBP1) and CTBP2, which are members of a different co-repressor family and are recruited independently of the NCoR and SMRT complexes[66] (FIG. 3a). Similarly, other co-repressor complexes, such as the transducin-like enhancer of split 1 (TLE1) complex, which contains poly(ADP-ribose) polymerase 1 (PARP1), include components that are specifically required to act as sensors and to mediate dismissal of the co-repressor complex from promoters[67,68].

Co-repressor complexes that contain NCoR, SMRT and HDACs are not only associated with nuclear receptor-mediated repression but can also be recruited to the promoters of inflammation-associated genes that are regulated by the transcription factors NF-κB, activator protein 1 (AP1) and TEL (an ETS factor also known as ETV6). Therefore, it is not surprising that the clearance of co-repressors from these promoters is also a prerequisite for promoter activation by these transcription factors. Interestingly, the same co-repressor complexes are not only required for active repression of inflammatory target genes in the absence of stimulus but can also be responsible for ligand-dependent transrepression of those same genes by PPARγ and liver X receptors (LXRs). In this context, it is the clearance of the co-repressors from pro-inflammatory genes that can be specifically prevented by the liganded nuclear hormone receptors, which suggests that fine regulation of the dynamics of cofactor exchange may be important for the modulation of the transrepressive effects mediated by certain natural and synthetic agonists[69,70] (FIG. 3b). In addition, clearance of NCoR from different sets of target genes was observed in response to inflammatory stimulation of distinct Toll-like receptors by a phosphorylation-dependent regulation of the TBL1 and TBLR1 exchange machinery. This is in accordance with the model in which the active dismissal of the co-repressor complexes is an important layer of regulation that helps to shape gene-specific transcriptional responses[71] (FIG. 3c).

Exceptions to the rule: ligand-dependent co-repressors and regulated cofactors. When considering any model of cofactor action, it must be noted that not all co-repressors have the same mechanisms of recruitment. For example, nuclear receptor-interacting protein 1 (NRIP1, also known as RIP140), ligand-dependent co-repressor (LCOR), preferentially expressed antigen in melanoma (PRAME), prohibitin 2 (PHB2) and transcription intermediary factor 1α (TIF1α, also known as TRIM24) are all ligand-dependent co-repressors that are recruited to the DNA-bound liganded receptor by classic LxxLL active motifs. However, they are characterized by specific repression domains that enable them to recruit more conventional co-repressors, such as CTBPs, HDACs or polycomb factors[72–77]. Whether the ligand-dependent co-repressors are also dismissed from the regulated promoter by an active process is not known. However, we can speculate that being able to switch from co-repressor to co-activator binding while in the presence of a ligand might be as important as switching from co-repressors to co-activators when a ligand binds. Regulation of

Transrepression
Transcriptional repression mediated by transcription factors that are not directly bound to DNA but are recruited to the promoter by other transcription factors.

such a process may be important in modulating different phases of the kinetics of gene activation in response to hormonal stimulation. For a more detailed analysis of the known mechanisms of action of agonist-bound co-repressors, we refer readers to specific reviews[78,79].

In addition to 'conventional' co-repressors that are recruited to DNA by unliganded nuclear receptors and 'unconventional' co-repressors that are specifically recruited by ligands, there are multifunctional co-repressors. For example, the co-regulator hairless can either act

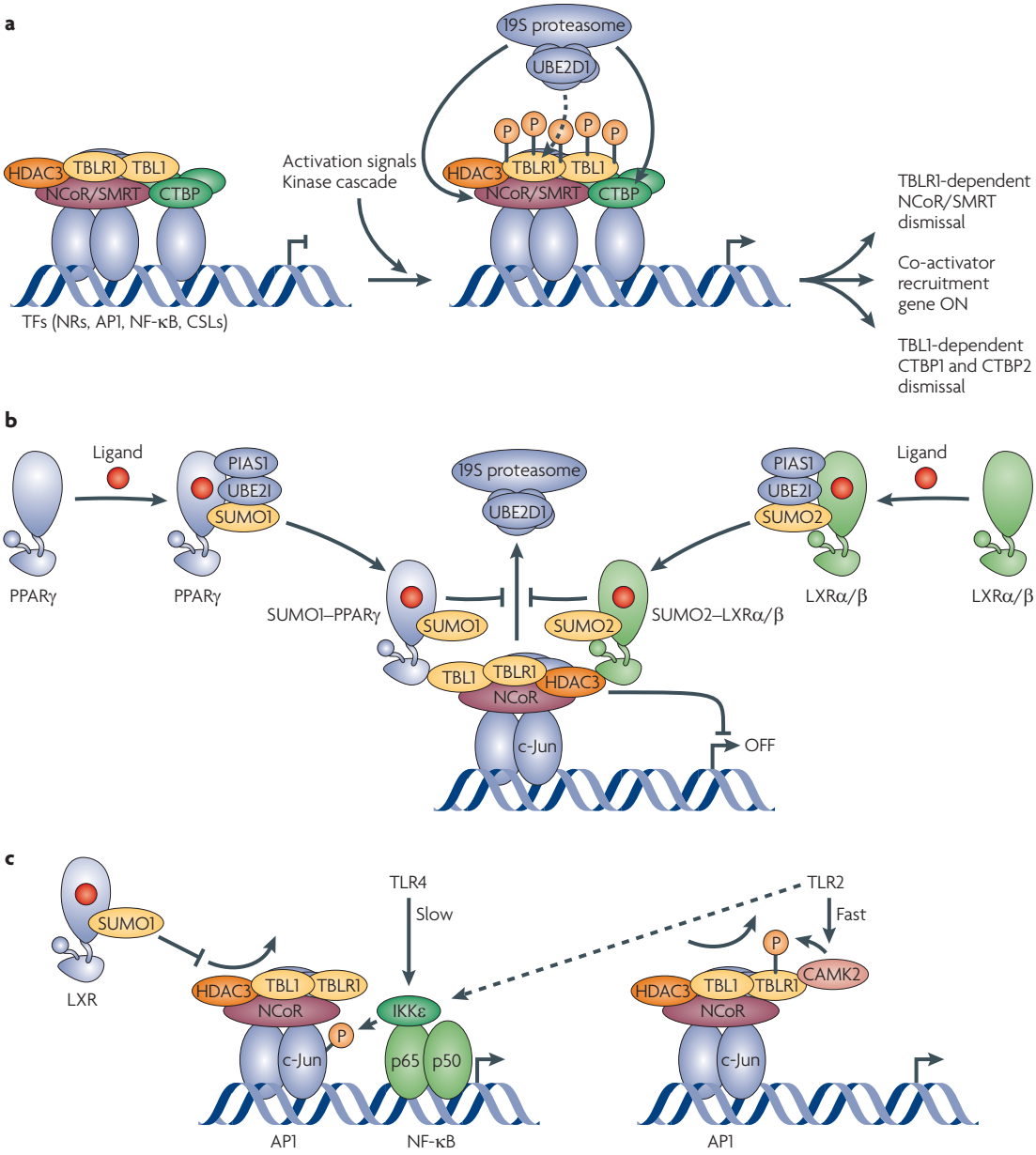

Figure 3 | **Co-repressor clearance by protein ubiquitylation. a** | Mechanism of nuclear receptor co-repressor or silencing mediator of retinoic acid and thyroid hormone receptor (NCoR/SMRT) and carboxy-terminal-binding protein (CTBP) clearance by the nuclear co-repressor exchange (NCoEx) factors transducin β-like 1 (TBL1, also known as TBL1X) and TBL-related 1 (TBLR1, also known as TBL1XR1). The 19S proteosome is recruited to the promoter by TBL1 and TBLR1 (dashed arrow), where it mediates ubiquitylation-mediated dismissal of the NCoR or SMRT complex and CTBP (solid arrows). **b** | Inhibition of the TBL1- and TBLR1-dependent co-repressor clearance by peroxisome proliferator-activated receptor-γ (PPARγ) and liver X receptor (LXR) transrepressive activities. **c** | Toll-like receptor (TLR)-specific NCoR clearance mechanisms in innate immune responses. AP1, activator protein 1; CAMK2, calmodulin kinase 2; CSLs, CBF1, suppressor of hairless and LAG1-like proteins; HDAC3, histone deacetylase 3; IKKε, IκB kinase ε; NF-κB, nuclear factor-κB; NRs, nuclear receptors; P, phosphorylation; PIAS1, protein inhibitor of activated STAT protein 1; UBE2D1, ubiquitin-conjugating enzyme E2 D1; UBE2I, ubiquitin-conjugating enzyme E2 I. Part **a** is modified, with permission, from REF. 66 © (2008) Elsevier. Part **b** is modified, with permission, from REF. 70 © (2007) Elsevier. Part **c** is modified, with permission, from REF. 71 © (2009) Elsevier.

as a conventional co-repressor of unliganded thyroid hormone receptor (THR) or form a repressive complex with the retinoic acid receptor (RAR)-related orphan receptors (RORs) and the vitamin D3 receptor (VDR) in an agonist-dependent fashion[80]. There are also examples of cofactors, such as KRAB-associated protein 1 (KAP1, also known as TIF1β and TRIM28), that can work as either co-activators or co-repressors[81]. These observations suggest that the classification of cofactors as co-repressors or co-activators cannot be considered too rigidly.

In conclusion, although we can make some broad generalizations about the mechanisms of recruitment, dismissal and action of cofactor complexes, the complexity that has been revealed suggests that each case needs to be investigated in a cell-specific and context-specific manner to understand how co-repressors are dynamically regulated and mediate specific repressive effects.

Biological roles of co-repressors

The models of co-repressor function discussed above set out some general principles that might underlie gene repression. But what are the functions of co-repressor complexes in the context of the organism as a whole? Are different complexes functionally equivalent? And what are the consequences of their misregulation? In this section, we consider what has been learnt of the biological functions of co-repressors, focusing on NCoR, SMRT and HDACs by way of example.

Mammalian models. Over the past several years, genetic studies in mice have provided new insights into the function and specificity of co-repressor proteins in mammalian development. TABLE 2 summarizes the phenotypes of these mouse models. Based on these models, it seems that there is no general rule for the consequences of co-repressor mutation. In some cases, gene knockout results in early embryonic lethality, whereas in others there are no apparent phenotypic consequences. For some gene families, such as the sirtuin family, the lack of profound phenotypes may be due to redundancy between related factors. In other cases of closely related factors, such as SIN3A and SIN3B, one factor seems to be more essential than the other, despite their overlapping expression patterns. Modulation of co-repressors by distinct signalling pathways may also account for differences between closely related proteins; for example, NCoR but not SMRT can be phosphorylated by the AKT1 kinase, resulting in translocation of NCoR from nucleus to cytoplasm (FIG. 4a). For methyl-CpG-binding domain protein 2 (MBD2) and MBD3, differences between their interacting partners seem to be responsible for the notable differences in embryonic developmental phenotypes caused by their mutation. This might also be true for NCoR and SMRT, which bind preferentially to THRs and RARs, respectively (FIG. 4b). Therefore, it is difficult to make any generalizations regarding the role of co-repressor proteins in mammalian development. Here, we consider in more detail the biological roles of NCoR and SMRT to show the multiple consequences of co-repressor gene deletion, as they can provide representative examples of the above explanations for phenotypic outcomes.

In early embryogenesis, absence of either NCoR or SMRT seems not to affect development, possibly owing to the fact that both factors are highly expressed early in embryogenesis (K.J. and M.G.R., unpublished observations) and can therefore compensate for the absence of one another. Although the removal of either gene results in lethality at mid-gestation (~embryonic day 14.5), the organ systems affected by mutation of *Ncor1* (which encodes NCoR) or *Smrt* (also known as *Ncor2*) seem to be quite distinct: NCoR seems to be crucial in the development of erythrocytes and thymocytes, whereas SMRT is required for late development of the embryonic heart[82–84]. At least in thymocytes, there is evidence that this specificity results from distinct expression patterns, as NCoR was detected in thymocytes but SMRT was not present (although it is found in thymic stroma)[83].

Differential expression in the developing brain may also have phenotypic consequences; *Smrt* mRNA is primarily expressed in the ventricular zone region, where multipotent neural precursors reside, whereas *Ncor1* is expressed at much lower levels in the ventral telencephalon, including the ventricular zone (FIG. 4c). Both NCoR and SMRT have been implicated in regulation of embryonic neural stem cell (eNSC) proliferation and differentiation, in which they have important roles in controlling neural stem cell maintenance and lineage decisions[62,84]. eNSCs that lack NCoR fail to self-renew and differentiate prematurely down the astroglial pathway, whereas SMRT-deficient eNSCs prematurely differentiate down both the astroglial and neuronal pathways (FIG. 4d). Regulation of the astroglial lineage by these co-repressors seems to be dose-dependent rather than factor-specific, as eNSCs cultured from mice that are heterozygous for both NCoR and SMRT deletions also prematurely differentiate down the astroglial lineage. Numerous biochemical studies have suggested that RAR preferentially recruits SMRT, whereas THR preferentially recruits NCoR (FIG. 4b). Differentiation down the neuronal pathway in the absence of SMRT was found to depend on RAR, so this case might be an example of how distinct interacting proteins can regulate the biological function of co-repressors (FIG. 4d). Furthermore, in eNSCs in which the *Smrt* gene has been deleted, neuronal differentiation is regulated at least in part by the retinoic acid (RA)-responsive JMJD3, which is itself an epigenetic regulator and a member of the JMJC family of putative histone demethylases[85]. JMJD3 and its homologous factor, lysine-specific demethylase 6A (KDM6A, also known as UTX), are H3K27me3 demethylases[86–91] that function to oppose H3K27 trimethylation by enhancer of zeste 2 (EZH2), which is the enzymatic component of PRC2 (REF. 92) and has a well-established role in stem cell differentiation (reviewed in REF. 93). H3K27me3 has been associated with pluripotency[94], and the findings of several recent genome-wide studies implicate H3K27me3 as a dynamic mark that reflects developmental potential in specific cell lineages and developmental stages[53,95–97]. Therefore, preventing

Telencephalon
The anterior portion of the forebrain, which consists of the cerebral cortex, basal ganglia, corpus striatum and olfactory bulb.

Astroglia
Astrocytes (collectively known as astroglia) are star-shaped glial (non-nervous) cells in the brain and spinal cord that surround and support neurons and are now additionally thought to have a number of active roles in the brain.

neuronal differentiation in eNSCs relies on the interplay of at least two distinct classes of co-repressors, providing another layer of complexity in the interpretation of biological outcomes.

Domain-specific studies. One way to discern which co-repressor-interacting partners influence particular biological outcomes might be to genetically modify the interaction domains of the co-repressors. Interestingly,

Table 2 | ***In vivo* models of co-repressor factors**

Gene	Phenotype*	Refs
Baf180	Embryonic lethality; cardiovascular	144
Baf250a	Early embryonic lethal; germ-layer formation	145
Baf53b	Postnatal lethality; neurogenesis	146
Bmi1	Posterior transformation; defects in haematopoietic stem cell renewal	147
Brg1	Early embryonic lethal; peri-implantation stage	148
Brm	Normal development	149
Chd4	T cell development	150
Ctbp1	Small, some postnatal lethality	151
Ctbp2	Early embryonic lethal; extra-embryonic vascularization; cardiovascular	151
Eed	Early embryonic lethal; anterior–posterior patterning defects	152,153
Ezh2	Early embryonic lethal	154
Hdac1	Embryonic lethal	155
Hdac2	Perinatal lethal; cardiovascular	156
Hdac3	Early embryonic lethal	104,105
Hdac4	Postnatal lethal	157
Hdac5, *Hdac6*, *Hdac9*	Normal development	158,159,160
Hdac7	Embryonic lethal; vascular defects	161
Hdac8	Perinatal lethal; skull morphogenesis	162
Kap1	Early embryonic lethal	163
Lsd1	Early embryonic lethal; gastrulation	139
Mbd2	Normal development	164
Mbd3	Early embryonic lethal	164
Mta2	Partial embryonic lethality; autoimmune disorder	165
Ncor1	Embryonic lethal; erythropoesis	83
Parp1	Develop normally	166,167
Phc1, *Phc2*	Perinatal lethality; anterior–posterior patterning defects	168,169
Ring1	Develop normally; skeletal phenotype	170
Rnf2	Early embryonic lethal	171
Sin3a	Early embryonic lethal	172
Sin3b	Embryonic lethal	173
Sirt1	Embryonic and perinatal lethal	174–176
Sirt3–Sirt5	Normal development	177,178,179
Smrt	Embryonic lethal; cardiovascular	82
Suz12	Early embryonic lethal	180
Tab2	Embryonic lethal; liver degeneration	181
Tbl1, *Tblr1*	Early embryonic lethal	63
Zbtb33	Normal development	182

*Early embryonic lethal indicates lethality prior to embryonic day 10.5. Baf, BRG1-associated factor; *Brg1*, BRM/SWI2-related gene 1 (also known as *Smarca4*); *Chd4*, chromodomain helicase DNA-binding protein 4; Ctbp, carboxy-terminal-binding protein; *Eed*, embryonic ectoderm development; *Ezh2*, enhancer of zeste 2; Hdac, histone deacetylase; *Kap1*, KRAB-associated protein 1 (also known as *Tif1β* and *Trim28*); *Lsd1*, lysine-specific histone demethylase 1; Mbd, methyl-CpG-binding domain protein; *Mta2*, metastasis-associated 2; *Ncor1*, nuclear receptor co-repressor 1; *Parp1*, poly(ADP-ribose) polymerase 1; Phc, polyhomeotic-like; *Ring1*, ring finger protein 1; *Rnf2*, ring finger protein 2; Sirt, sirtuin; *Smrt*, silencing mediator of retinoic acid and thyroid hormone receptor (also known as *Ncor2*); *Suz12*, suppressor of zeste 12; *Tab2*, Tak1-binding protein 2 (also known as *Map3k7ip2*); *Tbl1*, transducin β-like 1 (also known as *Tbl1x*); *Tblr1*, transducin β-like-related 1 (also known as *Tbl1xr1*); *Zbtb33*, zinc finger and BTB domain-containing 33 (also known as Kaiso).

a knock-in of a mutated form of the nuclear hormone receptor interaction domains of SMRT (SMRT^{mRID}) did not recapitulate the heart and brain development phenotypes observed for the knockout of SMRT. Rather, these mice survived to adulthood but had widespread metabolic defects, including reduced respiration and/or energy consumption, insulin resistance and increased adiposity[98]. Mice in which the nuclear hormone interaction domain of NCoR was specifically deleted in the liver also revealed a role for NCoR in metabolism[99]. Similarly, conditional deletion of HDAC3 in liver disrupted metabolic transcriptional networks, which is consistent with biochemical data that have shown NCoR and HDAC3 to be in the same complex[100]. Notably, when wild-type NCoR was replaced by a mutant (NCoR DADm) that is unable to bind HDAC3, the mutant mice survived, which suggests that NCoR-recruited HDAC3 activity is dispensable or is compensated for during development[101].

Nuclear hormone receptors have well-established roles during development (for reviews, see REFS 102,103), and gene deletion of *Hdac3* results in early embryonic lethality[104,105]; therefore, the lack of developmental phenotypes observed for SMRT^{mRID} and NCoR DADm are surprising given the well-established connections of these co-repressors to nuclear hormone receptors and to HDAC3. Certain biological aspects of NCoR and SMRT repression have been attributed to other DNA-binding transcription factors. For example, in the developing heart, SMRT seems to cooperate with the forkhead family repressor FOXP1 (REF. 82), and NCoR has been suggested to repress activation of the astrocytic glial fibrillary acidic protein (*Gfap*) gene in eNSCs through interaction with the Notch pathway transcription factor recombination signal-binding protein for immunoglobulin κJ region (RBPJ)[62]. However, given the large body of literature that confirms the role of nuclear hormone receptors and their ligands in development, it seems unlikely that co-repressors are completely dispensable for these actions.

In *Xenopus laevis*, expression of a dominant-negative SMRT resulted in embryos that exhibited phenotypes that were similar to those of embryos treated with RA, and this study showed that repression of RAR signalling is required for head development[106]. Studies in *X. laevis* have also confirmed a role for in THR-mediated developmental progression (reviewed in REF. 107). Furthermore, interaction of the ecdysone receptor with the fly SMRT analogue, SMRTER, is crucial for proper development in *D. melanogaster*[16]. Therefore, rather than 'redesigning' the role of nuclear hormone receptor transcriptional repression in development, it seems likely that mammals have evolved a redundancy within the system to protect proper development. It may be that functional redundancy between NCoR and SMRT — in terms of their role in repression by nuclear hormone receptors during development — allows for compensation for the loss of either protein. This might also explain the absence of a developmental phenotype for NCoR DADm mice. Genetic experiments in which both NCoR and SMRT are mutated are needed to clarify the role of these co-repressors in mediating aspects of development associated with nuclear hormone receptors and HDAC3.

Some of the mild phenotypes described above might result from an ability of other HDAC proteins to compensate for the absence of HDAC3 activity, even though genetic models of HDAC loss of function have been shown to be mostly non-redundant (for a review, see REF. 108). Also, although the function of the nuclear hormone-binding domains of NCoR and SMRT has been characterized in adult mice, the embryonic lethality of the null mutants has precluded analysis of these proteins beyond the embryo. Therefore, conditional knockout models for NCoR and SMRT will be essential for future *in vivo* analysis.

Disease. Co-repressors of transcription have been implicated in various diseases (reviewed in REF. 109), as might be expected from their extensive role in integrating numerous biological pathways. NCoR and SMRT have been implicated in resistance to thyroid hormone (RTH), a human genetic disease that is characterized by an impaired physiological response to thyroid hormone. RTH patients carry mutations in THRβ that result in failure to release NCoR or SMRT from this receptor upon hormone treatment[110,111]. Therefore, an understanding of the function of the co-repressor provides a mechanistic explanation for the clinical observation.

NCoR and SMRT have also been linked to several types of leukaemia, including acute promyelocytic leukaemia, acute myeloid leukaemia and a form of paediatric B-cell acute leukaemia (for a review, see REF. 112). These leukaemias are often caused by different translocation events that pair co-repressor-interacting proteins with proteins that are not normally regulated by NCoR or SMRT. This results in aberrant gene repression of target pathways, which can be overcome in some cases by HDAC inhibitors[113]. These leukaemias are characterized by the presence of undifferentiated haematopoietic cells, which is consistent with the hypothesis that was proposed based on animal model studies (discussed above) that NCoR and SMRT have a role in maintaining eNSCs in an undifferentiated state.

Recently, a correlation between NCoR expression and the astrocyte-derived cancer glioblastoma multiforme (GBM) — the most common and aggressive type of primary brain tumour — has been observed, and a connection between NCoR and so-called GBM cancer stem cells has been proposed[114,115]. In recent years, the concept of cancer stem cells has been intensely studied, and the similarities between so called tumour-initiating cells and stem cells in the nervous system has been recognized[116]. This has resulted in an interest in understanding the similarities and differences in the characteristics of eNSCs compared with central nervous system tumour cells. Intriguingly, protein screening using clinical samples has shown that nuclear NCoR is dramatically increased in severe grades of astrocytomas, which correlates with loss of *GFAP* expression, upregulation of nestin and progress from WHO (World Health Organization) grade II to grade IV glioma[114,117]. In addition, it was recently shown that inhibition of the NCoR pathway by simultaneous administration of RA and the protein phosphatase 1 (PP1)

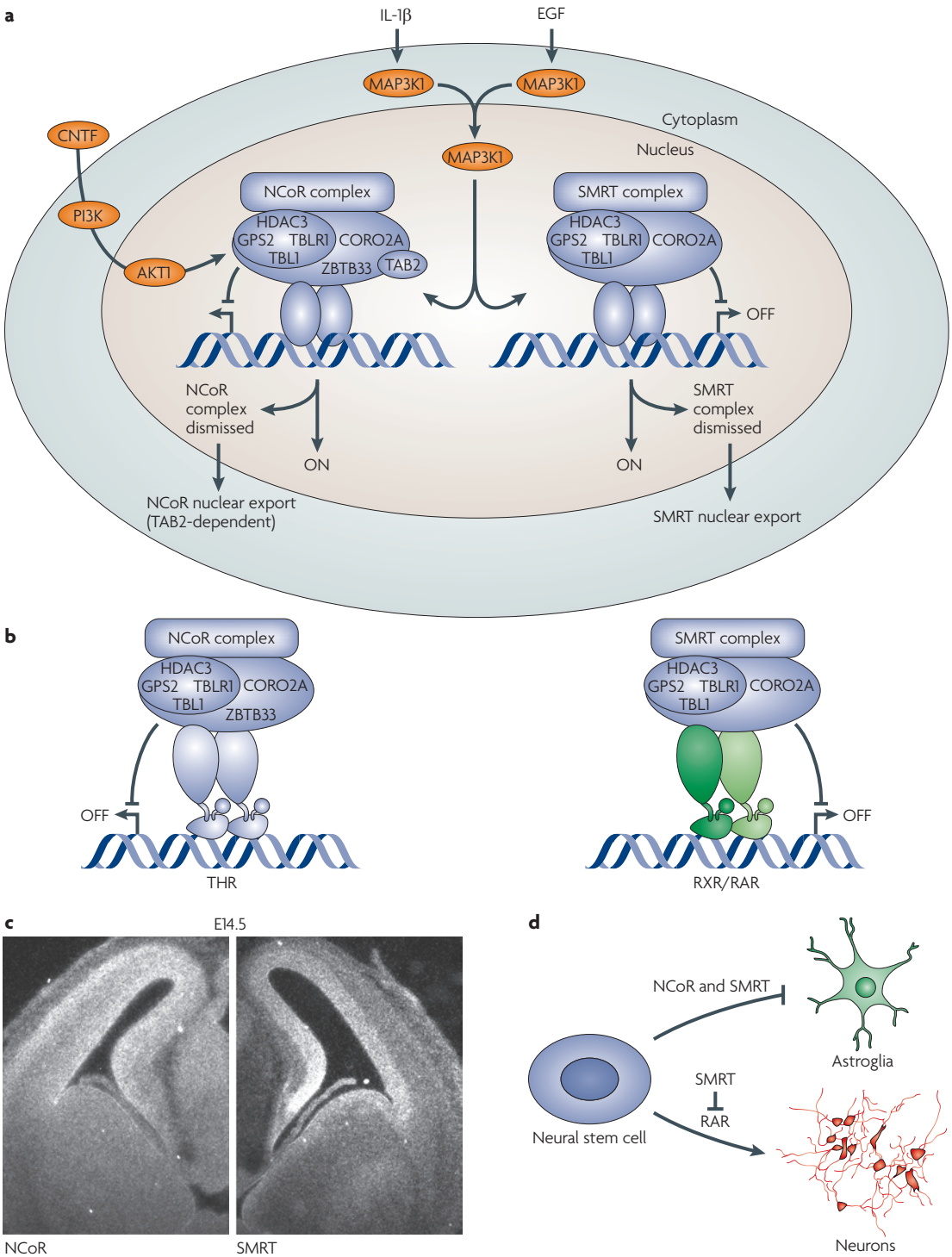

Figure 4 | **Differential aspects of NCoR and SMRT regulation and function. a** | Nuclear receptor co-repressor (NCoR, also known as NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT, also known as NCOR2) are regulated by different kinase pathways[37,61,62,123,124]. **b** | NCoR and SMRT show differential preferences for DNA-binding transcription factors. NCoR favours the thyroid hormone receptor (THR) and SMRT favours the retinoic acid receptor (RAR)[44,125–128]. **c** | Cell type-specific expression differs between NCoR and SMRT[83,84]. Shown here is the *in situ* analysis of a wild-type embryonic day (E)14.5 mouse cortex using RNA antisense probes for *Ncor1* (which encodes NCoR) and *Smrt*[84]. **d** | In the regulation of embryonic neural stem cells, both NCoR and SMRT are required to repress a glial fate, but only SMRT is required to repress neuronal differentiation through the RAR pathway. CNTF, ciliary neurotrophic factor; CORO2A, coronin 2A (also known as IR10); EGF, epidermal growth factor; GPS2, G-protein-pathway suppressor 2; HDAC3, histone deacetylase 3; IL-1β, interleukin-1β; MAP3K1, mitogen-activated protein kinase kinase kinase 1; PI3K, phosphoinositide 3-kinase; RXR, retinoid X receptor; TAB2, TAK1-binding protein 2 (also known as MAP3K7IP2); TBL1, transducin β-like 1 (also known as TBL1X); TBLR1, transducin β-like-related 1 (also known as TBL1XR1); ZBTB33, zinc finger and BTB domain-containing 33 (also known as Kaiso).

inhibitor okadaic acid is sufficient to induce a dramatic increase in differentiation and inhibition of growth in GBM cells[115]. Although results from specific ablation of NCoR and/or SMRT in gliomas are lacking, these reports suggest that NCoR and SMRT may be putative targets for glioblastoma therapy.

Circumstantial evidence has also linked NCoR and/or SMRT expression and subcellular localization to various other cancers, including colorectal carcinoma[118] and endometrial carcinoma[119,120]. It will be interesting to determine with further analysis whether the mechanistic role of co-repressors in these cancers is also related to their role in preventing initiation of differentiation programmes.

Future directions

Although our view of transcriptional co-repressors and their role in asserting proper gene expression outcomes has evolved considerably over the past decade, knowledge, and perhaps appreciation, of the crucial role of gene repression still lags behind that of gene activation. The continued use of next-generation sequencing and genome-wide approaches to reveal transcriptional outcomes should help to illuminate many of the unknowns. In this Review, we have discussed how recently emerged data have begun to change our understanding of the actions of co-repressors; however, there are still many questions that remain to be answered. For instance, a picture of the genome-wide binding patterns of all known co-repressors would be useful, coupled with a genome-wide view of the dynamics of their responses to treatment with ligands or other signals. Moreover, the current literature lacks details of the specificity of histone deacetylation. As HDAC inhibitors have been shown to be promising treatments for various solid tumours and haematological cancers, as well as for other diseases (for a review, see REF. 108), it would be of great interest to understand the mechanistic details of how these proteins work, and to determine whether they provide the histone code with another layer of specificity. Another interesting aspect is that a three-dimensional view of the nucleus can be provided by chromosome conformation capture-based methods (known as 3C and 4C), and such studies will be instrumental for understanding mechanisms by which nuclear neighbourhoods influence gene repression (for reviews, see REFS 121,122). In addition, they might illuminate previously unrecognized synergy between distinct classes of co-repressors. Finally, defining the roles of the individual functional domains of different co-repressor proteins in specific *in vivo* models would also be useful for elucidating the roles of gene repression in models of disease.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

UniProtKB: http://www.uniprot.org
CORO2A | CTBP1 | CTBP2 | GPS2 | HDAC3 | LCOR | MAP3K7IP2 | NCOR1 | NCOR2 | NRIP1 | TBL1X | TBL1XR1 | ZBTB33

FURTHER INFORMATION

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