TET proteins, New Cofactors for Nuclear Receptors

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Devant le jury composé de :

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How time flies, I have been in France for 4 years. My first day beginning this 4-years adventure is still vivid in my mind. September 12, 2013, it was a sunny day. On my way to ENS, I kept talking with the taxi driver with my awkward French, I was too excited to feel tired after 11 hours flight. Of course, the uncertainty of this adventure also upsets me. Will everything goes well in this foreign country; will I succeed my PhD at the end? Now, I could tell the past self that everything is fine, because you have never been alone. At the very end of my PhD and at the beginning of my thesis, I would like to express my gratitude to everyone who has ever helped me; it is your company that makes this 4-years-time an invaluable treasure for me.

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Résumé

L'hormone thyroïdienne (T3) contrôle à la fois les processus développementaux et physiologiques. Elle agit via les récepteurs de l'hormone thyroïdienne (TR), membres de la famille des récepteurs hormonaux nucléaires. Ils agissent comme des facteurs de transcription dépendants du ligand. La méthylation de l'ADN en position 5 de la cytosine est une modification épigénétique importante qui affecte la structure de la chromatine et l'expression des gènes. Des études récentes ont établi un rôle important des protéines de la famille TET (Ten-eleven translocation) dans la régulation de la dynamique de la méthylation de l'ADN. Elles convertissent la 5-méthyl-cytosine (5mC) en 5-hydroxyméthylcytosine (5hmC). D'autres études ont démontré que les protéines TET (TET1, TET2 et TET3) possèdent des fonctions de régulation transcriptionnelle dépendantes et indépendantes de leur activité catalytique. Notre étude a identifié TET3 comme une nouvelle protéine interagissant avec TR. Le domaine AF2 de TR ainsi que le domaine catalytique et le domaine CXXC de TET3 sont responsables de cette interaction. Celle-ci permet la stabilisation de TR lié à la chromatine, entraînant une potentialisation de son activité transcriptionnelle. L'effet de modulation de TET3 sur TR présenté ici est indépendant de son activité hydroxylase de TET3. Ainsi, cette étude met en évidence un nouveau mode d'action de TET3 en tant que régulateur non classique de TR, modulant sa stabilité et son accès à la chromatine plutôt que son activité de transcription intrinsèque. Des mutations du gène codant pour TRα provoquent le symptôme RTHα dont la gravité varie en fonction de la mutation. Les différentes capacités d'interaction des mutants TRα, pertinents pour la maladie de RTHα humaine, avec TET3 pourraient expliquer les différences d'effet dominant négatif. La fonction de régulation de TET3 pourrait s'appliquer plus généralement aux facteurs de transcription des récepteurs nucléaires, car différents membres de la superfamille des récepteurs nucléaires présentent la même interaction avec TET3, tels que AR (récepteur des androgènes), ERR (récepteur des œstrogènes) et RAR (récepteur de l'acide rétinoïque). L'interaction entre TET3 et RAR implique le domaine de liaison ADN de RAR. La pertinence fonctionnelle de l'interaction TET3 / RAR a été étudiée plus en détail dans les cellules souches embryonnaire (cellules ES). L'absence combinée des trois TET a entraîné la diminution de 5hmC et la dérégulation des gènes impliqués dans la différenciation des cellules ES. Parmi les gènes dérégulés, nous avons identifié un sous-ensemble de gènes cibles de l'acide rétinoïque, suggérant que les RAR (récepteurs d'acide rétinoïque) et les TET pourraient travailler ensemble pour réguler la différenciation des cellules ES. Une étude supplémentaire a révélé que les protéines TET peuvent jouer un rôle dans la facilitation du recrutement de RAR aux régions promotrices de ses gènes cibles. En outre, nos résultats montrent un rôle potentiel de l'activité hydroxylase des protéines TET dans la modulation de l'activité transcriptionnelle des RAR. En conclusion, notre travail a identifié les protéines TET comme nouveaux régulateurs des récepteurs nucléaires. Les mécanismes exacts impliqués doivent être étudiés plus avant.

Mots clés : récepteur d'hormone thyroïdienne (TR), méthylcytosine dioxygénase TET3, stabilité des protéines, recrutement à la chromatine, syndrome RTH, récepteur de l'acide rétinoïque (RAR).

Abstract

Thyroid hormone (T3) controls both developmental and physiological processes. Its nuclear receptors, thyroid hormone receptors (TRs), are members of the nuclear hormone receptor family which act as ligand-dependent transcription factors. DNA methylation at the fifth position of cytosine is an important epigenetic modification that affects chromatin structure and gene expression. Recent studies have established a critical function of the Ten-eleven translocation (TET) family proteins in regulating DNA methylation dynamics by converting 5-methyl-cytosine (5mC) into 5-hydroxymethylcytosine (5hmC). Studies demonstrated that TETs proteins (including TET1, TET2 and TET3) possess catalytic activity dependent and independent transcriptional regulatory functions. Our study identified TET3 as a new TR interacting protein. The AF2 domain of TR and the catalytic domain and CXXC domain of TET3 are responsible for their interaction. This interaction allows the stabilization of chromatin bound TR, resulting in a potentiation of its transcriptional activity. The modulation effect of TET3 on TR presented here is independent of its hydroxylase activity. Thus this study evidences a new mode of action for TET3 as a non-classical regulator of TR, modulating its stability and access to chromatin rather that its intrinsic transcriptional activity. Mutations in TRα cause the RTHα symptom which severity varies with the particular mutation. The differential ability of different TRα mutants, relevant for the human RTHα disease, to interact with TET3 might explain their differential dominant negative activity. The regulatory function of TET3 might be more general towards the nuclear receptor transcriptional factors since different members of the superfamily present the same interaction with TET3, such as AR (androgen receptor), ERR (Estrogen-related receptor) and RAR (retinoic acid receptor). The interaction between TET3 and RAR involves the DNA binding domain of RAR. The functional relevance of TET3/RAR interaction was further studied in ES cells. Combined deficiency of all three TETs led to depletion of 5hmC and deregulation of genes involved in ES differentiation. Among the deregulated genes, a subset of RA response genes was identified, suggesting that RARs (retinoic acid receptors) and TETs might work together to regulate ES cell differentiation. Further dissection revealed that TET proteins may have a role in facilitating RAR recruitment to the promoter regions of these RAR target genes. Moreover, our results indicated a potential role of the hydroxylase activity of TET proteins in modulating RAR transcriptional activity. Altogether, our work identified TET proteins as new regulators of NR (Nuclear Receptors). The exact mechanisms involved need to be further studied.

Key words: Thyroid Hormone Receptor (TR), Methylcytosine dioxygenase TET3, Protein Stability, Chromatin recruitment, RTHα syndrome, Retinoic acid receptor (RAR).

摘要

甲状腺激素(Thyroid Hormone，TH)的生理作用十分广泛，影响机体的生长发育、组织分化、物质代谢，并涉及到心脏、神经系统等多系统的功能。甲状腺激素的生物学功能主要是通过与甲状腺激素受体（TR）结合，进而调控下游靶基因的表达。甲状腺激素受体属于核激素受体（NR）家族，由 TRα 和 TRβ 基因（*THRA, THRB*）编码,主要包括 TRα1、TRβ1 和 TRβ2 三种亚型。目前研究表明 TR 在不同细胞类型中具有不同的靶基因，且这种细胞特异性的转录调控并非由 TR 在下游靶基因上的差异性结合造成的。因此其他因素，如细胞特异性转录辅助因子的表达，可能参与 TR 细胞特异性的转录调控。DNA 甲基化修饰作为真核生物细胞内一种重要的表观遗传修饰，能通过影响染色质结构，DNA 构象、稳定性等，起到调控基因表达的作用。近期研究发现了一种 DNA 去甲基化酶，TET(Ten-Eleven translocation)家族 DNA 羟化酶，能通过多种途径催化 5-甲基胞嘧啶(5-methylcytosine，5-mC)去甲基化从而调控 DNA 甲基化的平衡。研究表明 TET 家族蛋白（包括 TET1,TET2 和 TET3）具有羟甲基化酶活依赖性和非依赖性的转录调控功能。本课题鉴定出 TET3 为一个新的 TR 相互作用蛋白。TR 的 AF2 结构域和 TET3 的催化结构域（TET3-Cat）及 CXXC 结构域介导两个蛋白间的相互作用。这种相互作用增强了与染色质结合的 TR 的稳定性，从而正调控 TR 的转录活性。此外，TET3 对 TR 稳定性的调控是不依赖于 TET3 羟甲基化酶活性的。因此，这项研究揭示了 TET3 的一种新的作用模式，即作为 TR 的转录辅助因子，通过调控 TR 的蛋白质稳定性及 TR 与染色质的结合来调控 TR 下游靶基因的表达。甲状腺激素抵抗综合征(Resistance to Thyroid Hormone syndrom，RTH)是由于甲状腺激素受体基因突变，导致靶器官对甲状腺激素的敏感性降低，使得 TH 对全身组织器官作用障碍的一种罕见综合征，该综合征的严重程度随特定 TR 突变而变化。我们的研究发现不同的 RTH 相关的 TR 突变体与 TET3 的相互作用不同，且这种不同的相互作用导致 TET3 对 TR 突变体的稳定性的差异调节，从而差异性调控其显性负性作用（dominant negative effect）。我们的课题还鉴定出了 TET3 和雄性激素受体（androgen receptor，AR），雌性激素相关受体（Estrogen-related receptor，ERR）及视黄酸受体（retinoic acid receptor，RAR）的相互作用，暗示 TET3 可能对核激素受体蛋白具有普遍的调控作用。RAR 与 TET3 的相互作用是由其 DNA 结合结构域（DNA binding domain,DBD）介导的。我们又进一步在小鼠胚胎干细胞（ES）中研究了 TET3/RAR 的功能相关性。ES 细胞中 TET 家族蛋白的敲除导致部分 RAR 下游靶基因的异常表达并阻碍了 ES 细胞的正常分化，表明 RAR 和 TET3 可能协同作用进而调控 ES 细胞的分化。进一步的研究表明 TET 蛋白可能有助于 RAR 募集到这些 RAR 靶基因的启动子区域。此外，我们的结果表明 TET 蛋白的羟甲机化酶活性可能也参与了调节 RAR 的转录活性。总而言之，我们的工作鉴定出 TET 蛋白可作为 NR 辅助因子从而参与转录调控的新型作用模式，然而，TET 蛋白调控 NR 活性的确切机制仍有待进一步研究。

关键词：甲状腺激素受体（TR），TET(Ten-Eleven translocation)家族 DNA 羟化酶，蛋白质稳定性，染色质结合，甲状腺激素抵抗综合征(Resistance to Thyroid Hormone syndrom，RTH)，视黄酸受体（retinoic acid receptor，RAR）。

Abbreviations

5CaC: 5-carboxylcytosine

5fC: 5-formylcytosine

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

AF2: Activation Function domain 2

AR: Androgen Receptor

CRD: Cystine-Rich Domain

DBD: DNA Binding Domain

DNMT: DNA methyltransferase

DSBH: Double Strand β-helix Doamin

EB: Embryonic Body

ERR: Estrogen-receptor Related Receptor

HDAC: Histone Deacetylase

HDM: Histone Demethylase

hMeDIP: 5-hydroxymethylcytosine immunoprecipitation

HMT: Histone Methyltransferase

HRE: Hormone Response Element

LBD: Ligand Binding Domain

LSD1: Lysine Specific Demethylase 1

NCoR: Nuclear Receptor Co-repressor

NR: Nuclear Receptor

NuRD: Nucleosome Remodeling Deacetylase Complex

OGT: O-GlcNAc transferase

PRC2: Polycomb Complex 2

PRMT: Protein Arginine Methyltransferase

RA: Retinoic Acid

RAR: Retinoic Acid Receptor

RARE: Retinoic Acid Response Element

RTH: Resistance to Thyroid Hormone receptor

RXR: Retinoic X Receptor

SMRT: Silencing Mediator of Retinoic acid and Thyroid hormone receptor

SRC: Steroid receptor coactivator

TET: Ten-eleven Translocation

TH: Thyroid Hormone

TR: Thyroid hormone Receptor

TRE: Thyroid hormone Response Element

TRH: Thyrotropin-releasing Hormone

TSH: Thyroid Stimulating Hormone

Catalog

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1 Nuclear Receptor Superfamily

Cells of multicellular organisms need to communicate with each other to regulate their development and organize growth and cell division. Hormones contribute to these processes by acting as messengers between cells, telling them what is happening elsewhere and how they should respond. Fat-soluble hormones and intermediary metabolites, which can pass through the lipid bilayer of the cell membrane (Kushiro, T. et al, 2003), are well suited to mediate intercellular communications and to signal the status of the organism as a whole, such molecules have been exploited by nature to function as regulatory ligands for a major class of transcriptional regulators called nuclear receptors (NR). The 48 human members of this family include both receptors for which ligands are known and "orphan receptors" for which there are, as yet, no known ligands or no ligands (Germain P et al, 2006).

1.1 Sub-classification and Mechanisms of Signaling

Depending on the mechanisms of signaling, the nuclear receptor superfamily is sub-divided into three classes (Figure.1). Class I is the steroid receptor family, including the progesterone receptor (PR), the estrogen receptor (ER), the glucocorticoid receptor (GR), the androgen receptor (AR) and the mineralocorticoid receptor(MR). Steroid receptors are bound to heat shock proteins (HSPs), such as Hsp90 and Hsp70, in the cytoplasm. Ligand binding results in the dissociation of heat shock proteins, the free receptors then translocate to the nucleus and bind as homodimers to specific sequences of DNA known as hormone response elements (HREs). Steroid receptors bind to HREs consisting of two half-sites separated by a variable length of DNA, and the second half-site has a sequence inverted from the first (inverted repeat). Class II, or the thyroid/ retinoid family, includes the thyroid receptor (TR), vitamin D receptor (VDR), the retinoic acid receptor (RAR) and the ecdysone receptor (EcR). Class II receptors are retained in the nucleus regardless of the ligand binding status and in addition bind as hetero-dimers (usually with RXR) to DNA. In contrast to Class I receptors, the HREs of class II receptors are usually direct repeats with a core consensus sequence of the hexanucleotide "half-site" (A/G)GGT(C/A/G)A separated by a variable length of DNA, for example, TR binds to DR4(direct repeat separated by 4 nucleotides), RAR binds to DR2-5 and VDR binds to DR3(Umesono et al, 1991). Class II nuclear receptors are often complexed with corepressor proteins in the absence of ligand binding on DNA. Ligand treatment causes dissociation of corepressor and recruitment of coactivator proteins. Additional proteins including RNA polymerase II are then recruited to the NR/DNA complex that transcribes DNA into messenger RNA. The third class of nuclear receptors is known as the orphan receptor family. This class of nuclear receptor comprises a set of proteins sharing significant sequence homology to known nuclear receptors, but for which the ligands have not yet been identified or may not exist ("true orphans") or for which candidates have only recently been identified ("adopted orphans"). Members of "adopted orphan receptors" include retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR); "true orphan receptors" are like estrogen receptor-related (ERR), the chicken ovalbumin upstream transcription factors (COUP-TFs) and others (Figure. 1). And the modes of action of receptors in this subgroup are much diverse.

A

Class I	Class II	Class III	
Steriod Receptor	Thyroid/ Retinoid Receptor	Adopted Orphan Receptor	Orphan Receptor
PR	TR	RXR	COUP-TFs
ER	VDR	PPAR	ERR
GR	RAR	LXR	GCNF
AR	EcR	FXR	HNF4
MR		PXR/SXR	SF1
		CAR	LRH1
			DAX1
			SHP
			TLX
			PNR
			ROR

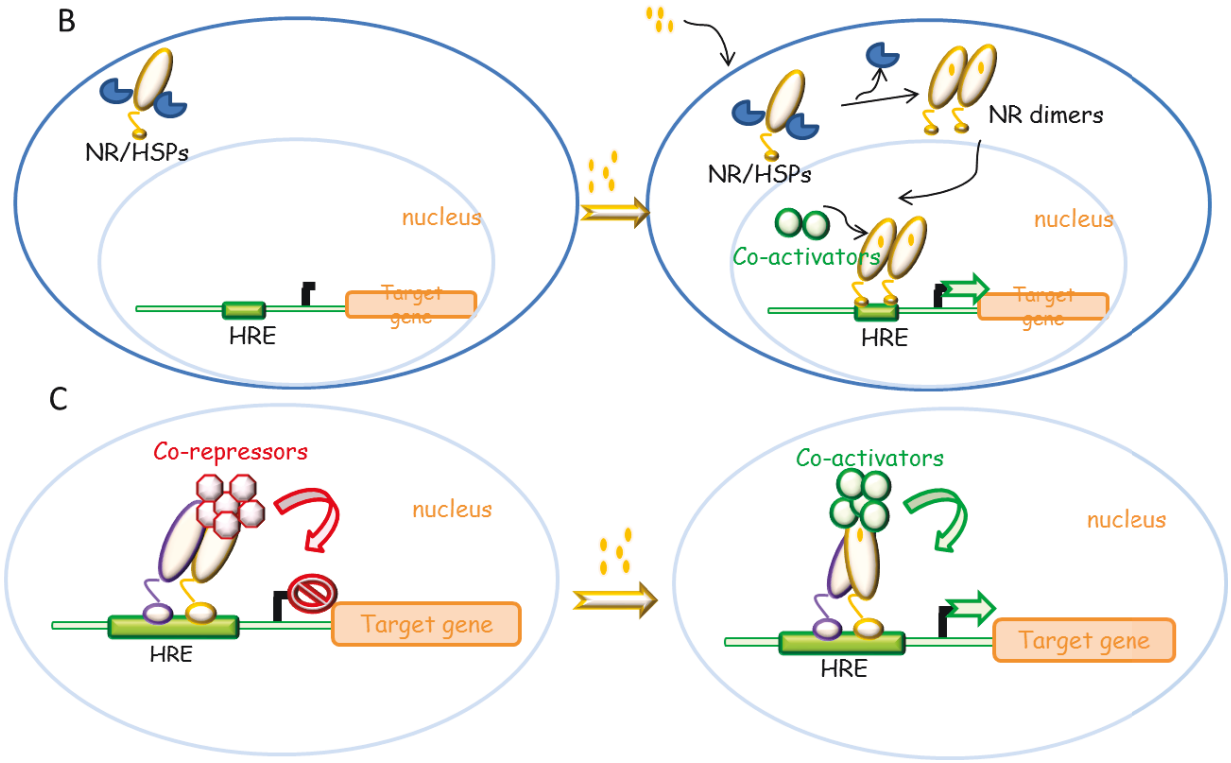


Figure 1 Classification and modes of action for different classes of nuclear receptors.

(A) Classification of some members of the nuclear receptor superfamily. Class I is the steroid receptor family; Class II is the thyroid/retinoic receptor, Class III is orphan receptor, including adopted and true orphan receptors. (B)Class I receptors (steroid receptors) include PR (progesterone receptor), ER (estrogen receptor), GR (glucocorticoid receptor), AR (androgen receptor) and MR (mineralocorticoid receptor). Class I receptors were captured in the cytoplasm by HSPs (heat shock proteins) in the absence of hormones, hormone binding induces the dissociation of HSPs and the translocation of receptors into the nucleus, and thus their binding to HREs (hormone response element). HREs of class I receptors are usually inverted repeats separated by a variable length of DNA. (C) Class II receptors (thyroid/ retinoid receptor family) include TR (thyroid receptor), VDR (vitamin D

receptor), RAR (retinoic acid receptor) and PPAR (peroxisome proliferator-activated receptor). They bind as hetero-dimers (usually with RXR) to DNA regardless of ligand binding, the HREs of class II receptors are usually direct repeats separated by a variable length of DNA.

1.2 Canonical Structure of Nuclear Receptors

Nuclear receptors share a common structural organization. The N-terminal region (A/B domain) Contains the activation function 1 (AF-1) whose action is independent of the presence of ligand (Wärnmark A. et al, 2003), A/B domains are highly variable and their 3D structure is not known. The DNA-binding domain (DBD, C domain) is the most conserved domain among nuclear receptors, the DBD contains a P-box, which is a short motif responsible for DNA-binding specificity and is involved in dimerization of nuclear receptors including the formation of both heterodimers and homodimers. The 3D structure of the DBD has been resolved for a number of nuclear receptors and contains two highly conserved zinc fingers. The structure represented (Figure.2) shows the DBD of the progesterone receptor DNA-binding domain dimer complexed with double stranded DNA (Roemer SC. et al, 2006). A linker region known as domain D is located between the DBD and the ligand binding domain (LBD). This region functions as a flexible hinge and contains a nuclear localization signal (NLS), which may overlap on the C domain, so it has Influences on NR intracellular trafficking and subcellular distribution. The largest domain is the C-terminal LBD (ligand binding domain), the LBD is moderately conserved in sequence and highly conserved in secondary structure among various nuclear receptors. The secondary structure of the LBD is referred to 12 α-helixes that are arranged around a central hydrophobic pocket, the exact structure of the pocket determines the type of ligand that will bind. Among the 12 a-helixes, the C-terminal most segment called helix 12 is the major architecture associated with ligand dependent activation function (AF-2), it can undergo dramatic shifts in position in response to the binding molecule in the pocket. Other α helices in the LBDs also shift in positions in subtle but still meaningful ways that can impact receptor activation. The AF-2 domain is necessary for recruiting transcriptional co-activators, which interact with chromatin remodeling proteins and the general transcriptional activation machinery. High resolution structures of full-length NRs on DNA have been determined for several nuclear receptors (Rastinejad F et al, 2016). Nuclear receptors may or may not contain a final domain in the C-terminus of the E domain, the F domain, whose sequence is extremely variable and whose structure and function are unknown.

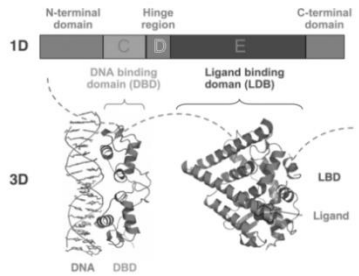


Figure 2 Structure Overview of Nuclear Receptors

A typical nuclear receptor consists of a variable N terminal region (the A/B domain), a highly conserved DNA-binding domain (DBD or C domain), a linker region (D domain), a moderately conserved C-terminal ligand binding domain (LBD or E domain) and with or without a C-terminus F domain.

1.3 Coregulator Codes of NR

1.3.1 Coactivator and Corepressor

Nuclear receptors directly activate or repress gene expressions by binding to hormone response elements (HREs) in promoter or enhancer regions of target genes. Their functions are directed by specific activation domains, referred to as ligand independent activation function 1 (AF-1) and ligand dependent activation function 2 (AF-2). The transcriptional activity of NRs is also regulated by a host of coregulatory proteins, referred to as coactivators or corepressors, respectively. Generally, ligand binding induces the exchange of corepressors to coactivators serving as the basic mechanism for switching gene repression to activation.

Coactivators

Coactivators are a group of proteins which are capable of potentiating nuclear receptor activity, these proteins always show a ligand dependent binding to nuclear receptors, the ligand-dependent recruitment of coactivators is mostly dependent on the AF-2 domain, which is the C-terminus helical segment within LBD (Huang P et al, 2010), though, a different set of coactivators is associated with the AF-1 domain. Biochemical and expression cloning approaches have been used to identify a large number of coactivators including chromatin remodeling complex, histone acetyltransferases, adaptor molecules, mediators and other proteins which have a role in shaping chromatin landscapes.

Chromatin Remodeling Complex. Chromatin remodeling is a fundamental process of chromatin reorganization. The chromatin state could be devided into heterochromatin and euchromatin state. The heterochromatin refers to a chromatin state with more condensed nucleosomal arrays, conversely, the normal nucleosomal array can be loosened, expose naked DNA in active chromatin states (termed euchromatin). Chromatin remodelers can induce the reversible changes of chromatin state through a process termed histone-octamer sliding (Saha A et al, 2006). Three types of ATP-dependent chromatin remodelers have been reported to facilitate NR mediated transactivation (Saha A et al, 2006), Switch/Sucrose non-fermenting (SWI/SNF complex), imitation switch (ISWI) complex and NURD/Mi-2/CHD complex. These complexes comprise core subunits and cell type specific regulatory subunits (Wu JI et al, 2007). Furthermore, several chromatin remodelers are direct coregulators for NRs. For example, the subunits of the SWI/SNF-type complex, BAF57 and WSTF, were respectively identified as direct interactor for ER and VDR (Belandia B et al, 2002; Kitagawa H et al, 2003). The association between NRs and these chromatin remodelers is believed to remodel nucleosomal arrays surrounding NR binding sites, and also to enhance accessibility of other NR coregulators.

Histone Acetyltransferases. Rates of gene transcription roughly correlate with the degree of histone acetylation. Acetylated residues are not limited to specific lysine residues; indeed, they are often seen on multiple sites on histone tails. Hyperacetylated regions is always associated with actively transcribed genes, whereas histone deacetylation is closely associated with inactivate chromatin states, suggesting that histone acetylation may play a critical role in overcoming repressive effects of chromatin structure on transcription (Handy DE et al, 2011). This concept was further supported by the finding that the

p/CAF and CBP/P300 (Chan HM et al, 2001), TIP60 complex (Brady ME et al, 1999) and GCN5/TRAPP complex (Yanagisawa J et al, 2002), that each possess intrinsic histone acetyltransferase (HAT) activity, are involved in NR mediated transcriptional activation, and the histone deacetylase (HDAC) family proteins have the reverse effect in transcriptional regulation (Taunton J et al, 1996).

Adapter Proteins. The steroid hormone receptor coactivator (SRC-1) was the first coactivator to be cloned that binds to nuclear receptors (Onate SA et al, 1995). SRC-1 belongs to the p160 family, other members of this family proteins known as TIF2/GRIP1, and p/CIP/A1B1/ACTR/RAC/TRAM-1 were identified subsequently. This family of proteins shares a common domain structure, the central domain contains three repeated motifs with a consensus sequence LXXLL (L represents leucine and X represents any amino acid), it is termed the NR box, which mediates ligand-dependent interactions with the nuclear receptor LBD (Perissi V et al, 2005); whereas the conserved C-terminal transcriptional activation domains mediate interactions with either CBP/p300 or protein-arginine methyltransferase. Interestingly, moderate HAT activity has also been attributed to SRC1 (Spencer TE et al, 1997). Furthermore, P160 family proteins have been suggested to function as coactivators, at least in part, by serving as adapter molecules recruiting CBP and/or p300 complexes to promoter-bound nuclear receptors in a ligand-dependent manner (Torchia J et al, 1997).

Mediator-like Complex. In addition to coactivator complexes that harbor nucleosome remodeling or histone acetyltransferase activities, other coactivator complexes have been identified. The best characterized of these is the TRAP (thyroid hormone receptor-associated protein)/DRIP (vitamin D3 receptor interacted proteins) /ARC (activator-recruited cofactor) complex, which serves as a bridge factor that could communicate the signals of DNA-bound NR with the basal transcription machinery. TRAP/DRIP/ARC complex exists as a large macromolecular complex containing at least 15 proteins that comprise a novel set of nuclear receptor coactivators. This complex is recruited to nuclear receptors in a ligand-dependent manner. Interestingly, they have been demonstrated to be required for transcriptional activation by nuclear receptors in cell-free in vitro transcription assays (Fondell JD et al 1996, Rachez Cet al, 1998). The DRIPs also enhance VDR activity on chromatin-organized templates despite an absence of HAT activity (Rachez Cet al, 1999), suggesting a potential unidentified chromatin remodeling function. Thus apart from their possible role to recruit RNA polymerase II holoenzyme to ligand-bound nuclear receptors, TRAP/DRIP/ARC complex might also contributes to remodelling chromatin, facilitating the organization of the pre-initiation complex or binding of other transcription factors (Fondell JD et al 1996, Rachez Cet al, 1998, Näär AM et al, 1999).

RNA and RNA processing proteins. The transcriptional co-activator PGC1 alpha (PGC1α) was initially identified as a peroxisome proliferator-activated receptor-gamma (PPARγ)-interacting proteinin a two-hybrid screen (Puigserver P et al, 1998). Subsequently, PGC1α was identified as a more general coactivator for NRs, including GR, TR, MR, ER, PPARα and ERR (Li S et al, 2008). PGC1α contains a N-terminal activation domain with the LXXLL motif (also called NR box) and a RNA-binding motif (RMM) and serine-arginine-rich (RS) region in the C-terminal region (Puigserver P et al, 2003). PGC-1α does not contain intrinsic enzymatic activities, rather a transcriptional activation domain in the N terminus then recruit other transcription regulators, such as CBP and SRC1 (Puigserver P et al, 1999). The TRAP/DRIP

mediator complex was also shown to dock at the C terminus of PGC1α protein, this interaction can aid in the displacement of repressor proteins such as histone deacetylases (HDACs) (Guan HP et al, 2005).This C-terminal region also interacts with the SWI/SNF chromatin-remodeling complex through its interaction with BAF60 (BRG1-associated factor 60a) component (Li S et al, 2008). The RNA-binding motif (RMM) and serine-arginine rich region (RS) domains in the C-terminus of PGC1α are characteristic of proteins with mRNA splicing and export activity. It has been found that PGC1 associates with RNA polymerase II during elongation and is also involved in the splicing and export of several mRNA products (Monsalve M et al, 2000), indicating that in addition to the ability of PGC-1α to serve as a docking site for different coactivators, it also functions to couple pre-mRNA splicing with the process of transcription.

The Steroid Receptor RNA Activator (SRA) was first identified by Lanz et al. in 1999 as a functional non-coding RNA able to co-activate steroid nuclear receptors. Further studies identified SRA as a broader co-regulator of nuclear receptors as well as other transcription factors (Lanz et al, 1999; Colley SM et al, 2009). Multiple studies have investigated the potential mechanisms of action of this peculiar RNA co-activator. It was first suggested to form a complex with SRC1 in vivo and selectively enhances the activity of steroids receptor via the AF-1 domain (Lanz et al, 1999), further study has proposed that this RNA acts also as a scaffold, embedded in ribonucleoprotein complexes recruited to promoters of target genes (Colley SM et al, 2009). Remarkably, SRA gene was later shown to be able to code for a protein referred to Steroid Receptor RNA Activator Protein (SRAP) (Emberley E et al, 2003). Even though the exact functions of SRAP remain to be fully elucidated, independent reports suggest that this protein also regulate steroid receptor signaling (Kawashima H et al, 2003; Chooniedass-Kothari S et al, 2006). Together, the SRA1 gene encodes both functional RNA and protein (SRAP) products, making it a unique member amongst the growing population of nuclear receptor coregulators.

Corepressors

NCoR/SMRT and Histone Deacetylases. Several members of the nuclear receptor family appear to be able to repress target gene expressions, alternatively functioning as a ligand dependent or independent repressor. This repressive effect is mediated by a group of corepressor proteins. The first identified nuclear receptor corepressors are NCoR (nuclear receptor corepressor) (Horlein AJ et al, 1995) and SMRT(silencing mediator of retinoid and thyroid hormone receptor)(Chen JD et al, 1995). NCoR or SMRT interacts with NR via the C-terminal NR box, that exhibit a consensus sequence of LXXI/HIXXX I/L (Perissi V et al, 2005). This motif is predicted to form an extended α helix, one helical turn longer than the LXXLL motif present in nuclear receptor coactivators. Moreover, they bind to the unliganded NRs close to where the coactivators bind, making a concomitant binding impossible.

The basal corepressors, NCoR and SMRT, function as platforms for the recruitment of several subcomplexes that often contain histone deacetylase activity. Consistently, NCoR and SMRT appear to be components of several distinct corepressor holocomplexes. Biochemical purification of NCoR or SMRT complexes has defined HDAC3, GPS2, and the transducin β-like factors TBL1 and TBLR1 as core components of larger NCoR/SMRT holocomplexes. The amino-terminal region of NCoR and SMRT contains the deacetylase activation domain (DAD) that interacts with and activates HDAC3, is required for their repressive roles. However, the repression by NCoR/SMRT could also be mediated by HDAC3-

independent mechanisms via TBL1, which interacts with hypoacetylated histones (Yoon HG et al, 2003). Furthermore, TBL1 and TBLR1 were shown to recruit specific ubiquitylation machinery, which is functionally required for the ligand dependent release of NCoR/SMRT by NRs (Perissi et al. 2004). Sin3A, HDAC1, 2, and the BRG1 complex, which were shown to be low-affinity components of NCoR/SMRT complexes (Ayer et al. 1995), also contribute to NCoR/SMRT-dependent repression. Sin3A/B and BRG1 complexes can probably contribute to the repression partially by stabilizing corepressor interactions with chromatin (Underhill et al. 2000).

A central issue in mediating repression by nuclear hormone receptors is the distinct or redundant function between corepressors NCoR and SMRT. Study shows that knockdown of SMRT had no effect on the recruitment of NCoR, and vice versa, suggesting that SMRT and NCoR are independently recruited to various target genes. However, overexpression of NCoR can restore repression of target genes after knocking down SMRT, implying a functional redundant role of NCoR and SMRT (Choi KC et al, 2008). Although NCoR and SMRT are highly homologous and the mode of action appears to be similar, NCoR has been implicated in many human diseases such as acute promyelocytic leukemia (He LZ et al, 1998), thyroid hormone resistance (Yoh SM et al, 1997) and thyroid carcinogenesis (Furuya F et al, 2007). It is not yet known whether SMRT is involved in human diseases. Moreover, the fact that NCoR knockout mice are embryonic lethal suggests that SMRT can't fully compensate for the functions of NCoR involved in development and survival (Jepsen K et al, 2000). Thus, all these observations suggest that two CoRs have both redundant and non-redundant biological functions.

Chromatin Remodeling Complex. Chromatin remodeling, a fundamental process of chromatin reorganization, appears to be indispensable for dynamic gene activation and also repression. The ATP-dependent chromatin remodelers imitation switch (ISWI)-type complexes are known to participate in both chromatin activation and repression (Ito T et al, 1997; Ito T et al, 1999). Moreover, Mi2-type chromatin remodeling complexes were shown to be potential corepressor for NRs, given that the transcriptional activity of liganded ERα can be suppressed by a cell cycle-dependent interaction with Mi2-type complexes (Okada M et al, 2008). The idea was further supported by the presence of HDACs in the Mi2-type complex (Zhang Y et al, 1998).

LCoR and RIP40. Hormone binding is normally associated with coactivator binding and subsequent transcriptional activation. A general model of receptor action where only coactivators are recruited to agonist-bound receptors, however, cannot account for all of the cofactors identified, since several corepressors, containing one or more NR boxes, exhibit ligand-dependent recruitment to NRs, exemplified by LCoR and RIP40.

RIP140 was initially characterized as a coactivator for estrogen receptor (ER) (Cavailles et al, 1995) that interacted with liganded receptors through its LXXLL motifs (Heery et al, 1997). However, subsequent work showed that RIP140 functioned as a corepressor for NRs (Lee et al, 1998; Miyata et al, 1998). LCoR (ligand-dependent repressor), was identified in a screen for proteins that interacted with liganded ERα. LCoR contains a single LXXLL motif that is critical for LCoR's agonist-dependent interaction with

receptors. Further studies showed that LCoR repressed hormone-dependent transactivation of several receptors in a dose-dependent manner (Fernandes et al, 2003).

Although the two proteins share only limited homology, LCoR and RIP140 have revealed remarkable functional parallels. One mechanism underline their ligand-dependent repression is their competition with p160 proteins for binding to agonist-bound LBDs, thus blocking transactivation (Eng et al, 1998; Fernandes et al, 2003). Supportively, structure studies have shown that p160 proteins, RIP140, and LCoR recognize the same LBD coactivator binding pocket formed in the presence of hormone, even though they do not make identical amino acid contacts (Henttu et al, 1997; Fernandes et al, 2003). LCoR and RIP140 were also shown to function as a molecular scaffold that recruits several proteins that function in transcriptional repression, including HDACs and the corepressor CtBP (corepressor terminal binding protein). Both interactions between LCoR/RIP140 and HDACs and CtBP were detailed studied (Wei et al, 2000; Vo et al, 2001; Fernandes et al, 2003).

Corepressors such as LCoR and RIP140 may function in negative feedback loops to attenuate hormone-induced transactivation, or function in hormone-induced target gene repression.

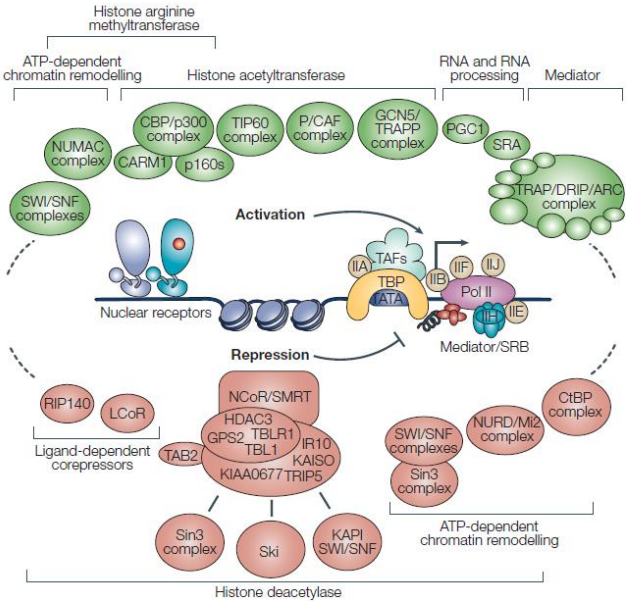


Figure 3 Coregulators for Nuclear Receptors (Perissi V & Rosenfeld MG et al, 2005).

The transcriptional activity of NRs is regulated by a host of coregulators, including coactivators and corepressors. Corepressors binding help to mediate gene repression, while ligand binding induces dissociation of corepressors and recruitment of coactivators, thus leads to gene activation. Coactivators include the P160 platform proteins (SRC-1, GRIP-1, p/CIP), the ATP-dependent chromatin remodeling complex (SWI/SNF), the histone acetyltransferases (CBP/P300, p/CAF, GCN5/TRAPP, Tip60), as well as factors that are involved in RNA processing (SRA) and the mediator complex (TRAP/DRIP/ARC). The corepressors include NCoR and SMRT, which function as platforms for the recruitment of several subcomplexes that often contain histone deacetylase activity; specific

corepressors, such as LCoR and RIP140, which are surprisingly able to recruit general corepressors on ligand induction; and the components of chromatin remodeling complex appear to be also involved in repression events.

1.3.2 NR coregulators Merge Transcriptional Coregulation with Epigenetic Regulation

As a result of enormous effort during the past two decades, a huge number of NR coregulators have been documented. The characterization of NR coregulators has contributed to our understanding of the molecular mechanisms of transcriptional control by NRs. NR coregulators were initially thought to serve as NR auxiliary regulators and/or adaptors, bridging NRs and basic transcription factors at target gene promoters, and efficiently forming stable complexes of transcription initiation machinery with RNA polymerase II (O'Malley BW et al, 2007). However, this idea was proven oversimplified given the diversity and versatility of coregulatory complex. Further functional dissection of NR coregulators revealed that their transcriptional coregulation was linked to epigenetic modifications, such as chromatin remodeling, histone modifications and DNA methylation.

Genomic DNA is packed into nucleosomes within chromatin, a nucleosome consists of 147bp DNA packed around a histone octamer, consisting of two H3-H4 histone dimers surrounded by two H2A-H2B dimers. H1 histone associates with the linker DNA located between the nucleosomes. The packaging and compaction of DNA by nucleosomes is dynamically changed during the process of transcription. The dynamic properties of nucleosomes are due to the action of nucleosome-modifying and -remodelling complexes. Modifying complexes, including histone modifiers and DNA methylation modifiers, add or remove covalent modifications at particular residues on the histone proteins or that on surrounding DNA, marks that are subsequently recognized by transcriptional regulators and other factors. Modifying complexes work in concert with chromatin-remodelling complexes, which restructure, mobilize or eject nucleosomes to regulate access to DNA (Saha A et al, 2006).

1.3.2.1 Chromatin Remodelers are NR Co-regulators.

Chromatin remodeling is a fundamental chromatin reorganization process that is essential for dynamic transcriptional control by NRs. Four chromatin remodeling complexes, known to move or to restructure nucleosomes in an ATP-dependent manner, have been identified: SWI/SNF, ISWI, NURD/Mi-2/CHD and INO80 family complexes. These four family complexes share similar ATPase domains but differ in the composition of their unique subunits (Ho L et al, 2010). And as we briefly discussed above, their roles in chromatin remodeling appear to be indispensable for both gene activation and repression.

SWI/SNF remodellers primarily disorder nucleosome positioning to promote transcription-factor binding and activation (Martens JA et al, 2003). However, it was also shown to facilitate transrepression by promoting the binding of transcriptional repressors. Repression by SWI/SNF complex is dependent on different subunits from those that confer transcriptional activation (Martens JA et al, 2002). Likewise, the ISWIa complex (Iswlp/Ioc3p) represses gene expression at initiation through specific positioning of a promoter proximal dinucleosome, however ISWIb complex (Iswlp/Ioc2p/Ioc4p) organize chromatin to promote transcriptional elongation (Morillon A et al, 2003). Mi-2 remodelers appear to associate with inactivating chromatin and gene repression, with HDACs as component of this complex (Zhang Y et al,

1998). The last chromatin remodeling complex, INO80 remodeler complex, has been characterized as a remodeler that has a pivotal role in DNA repair process (Morrison AJ et al, 2009).

Furthermore, direct interactions between chromatin remodelers and NRs were also identified. For example, the core subunit of SWI/SNF complex, BAF57, was shown to physically interact with ER, the ligand-dependent interaction between BAF57 and ER functions to target SWI/SNF complex to estrogen target genes thus mediating transactivation (Belandia B et al, 2002). Likewise, an interaction between VDR and a regulatory subunit of SWI/SNF, Williams syndrome transcription factor (WSTF), was also identified. WSTF was shown to coregulate VDR in both transcriptional activation and repression (Kitagawa H et al, 2003; Fujiki R et al, 2005). Thus the chromatin remodeling complex is believed to reset the nucleosomal arrays surrounding NRs binding sites, and to enhance accessibility of other NR coactivators or corepressors, depending on the promoter context.

1.3.2.2 Histone Modifiers are NR Co-regulators

Both histone modifications and nucleosomal structure are key factors defining the chromatin environment that are determinants for transcriptional controls. The histone tails stretched out of the nucleosome are more prone to post-transcriptional modifications (PTMs), including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADPribosylation, deimination and proline isomerization (Campos EI et al, 2009). Recent years, a number of novel types of PTMs have been discovered, including hydroxylation (Tan M et al, 2011; Unoki M et al, 2013), crotonylation (Kcr) (Tan M et al, 2011), N-formylation (Jiang T et al, 2007), succinylation (Xie Z et al, 2012), butyrylation (Chen Y et al, 2007) and O-GlcNAcylation (Sakabe K et al, 2010).

Among these histone modifications, the molecular link to transcriptional control is the best-established for histone acetylation. Histone acetylation is closely related to active chromatin state. In support of the role of histone acetylation in modifying chromatin structure, HATs are often viewed as NR coactivators, exemplified by p/CAF, CBP/P300, TIP60 complex and GCN5/TRAPP complex. In contrast, histone deacetylases (HDACs) activity are often necessary for NR corepressors complexes. As discussed above, HDAC3 are defined as core enzymatic component of large NCoR/SMRT corepressor complexes, furthermore, direct interactions between HDACs and/or their partner subunits in HDAC complexes with certain NRs were also identified. Moreover, the known NRs corepressors, LCoR and RIP140, were shown to mediate their repressive roles by recruiting HDACs (Wei et al, 2000).

Recent studies indicated that compared to histone acetylation, histone methylation is a more upstream modification governing chromatin state, this modification triggers orchestrated changes of the other histone-modification patterns to define chromatin state (Suganuma T et al, 2008). Histone methylation is dynamically regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs). Unlike histone acetylation, methylation occurs at specific lysine and arginine residues. Lysine residues can be mono-, di- or tri-methylated (Bannister and Kouzarides, 2004). Arginine can be mono-, symmetrically or asymmetrically di-methylated (Bedford and Richard, 2005). Histone methylation contributes to both transcriptional repression and activation. Methylation at H3K4 and H3K36 is typically linked to transcriptional activation, while H3K9 methylation is typically a repressive mark. Likewise,

histone arginine methylation can also be divided into activation marks, such as H4R3me2a, H3R2me2s, H3R17me2a, H3R26me2a, and repressive marks, such as H3R2me2a, H3R8me2a, H3R8me2s, H4R3me2s (Blanc RS et al, 2017).

Several proteins responsible for the methylation of specific residues have been characterized, catalyzing the transfer a methyl group from S-adenosyl methionine (SAM) to target lysine or arginine residues. All lysine methyltransferases but one of these contains a SET domain; they make up the SET-domain lyisne methyltransferase family. One exception is the DOT1 family, members of which methylate K79 in the globular region of histone H3 and which are structurally not related to SET-domain proteins (Feng Q et al, 2002). The protein arginine methyltransferases (PRMTs) fall into three categories according to their catalytic activity: type I (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6, and PRMT8) and type II (PRMT5 and PRMT9) enzymes carry out the formation of mono-methylated arginine as an intermediate before the establishment of symmetrically or asymmetrically di-methylated arginine, respectively (Yang et al., 2015). PRMT7 is a type III enzyme that catalyzes only the formation of mono-methylated arginine, and thus so far, histones are its only known substrates (Feng et al., 2013).

The histone lysine demethylases identified to date involve LSD1 (Lysine specific demethylase 1) and Jumonji C (JmjC) domain containing family proteins. LSD1, specific for mono- or di-methylated H3K4 (H3K4me1/2) or mono- or di-methylated H3K9 (H3K9me1/2), functions via FAD dependent oxidative reactions (amine oxidase family) (Y Shi et al, 2004; Metzger E et al, 2005). JmjC family proteins require Fe(II) and α-ketoglutarate as cofactors for oxidative hydroxylation. JmjC enzymes were identified possessing lysine demethylase activity with distinct methylation site and state specificities (Marmorstein R et al, 2009), as exemplified by JMJD1A, which was the first JmjC enzyme identified, showing demethylase activity towards H3K36me1/2 (Y Tsukada et al, 2006). However, the existence of arginine demethylases is still controversial (Yang and Bedford, 2013). A putative arginine demethylase, JmjD6, was identified but later shown to be a lysine hydroxylase (Webby et al., 2009). Recently, it was shown that certain lysine demethylases (KDM3A, KDM4E, KDM5C) also possess arginine demethylation activity *in vitro* (Walport et al, 2016), further investigation is required to confirm their functions *in vivo*.

Overall, the multiplicity of HMTs/HDMs and their different enzymatic properties appear to be necessary for the tightly regulated methylation state of chromatin. Given that histone methylation at different sites associate with different transcriptional outcome, it is reasonable that both HMTs and HDMs serve as coactivators and corepressors, depending on the amino acid residue targeted. Accumulating evidences suggest that these enzymes might serve as NR coregulators (table.1). Notably, lysine methyltransferases SUV39H1 and SETDB1 were reported to be co-repressors for NRs (Li J et al, 2002; Takada I et al, 2007), consistent with their role in catalyzing the formation of H3K9 metylation, which is typically a repressive mark. However, another two H3K9 methyltransferases, G9a and RIZ1 were shown to serve as coactivators for NRs (Lee DY et al, 2006; Carling T et al, 2004), further investigations suggested that their coactivator function is related to inhibited H3K9 methyltransferase activity by other associated transcriptional activators. A later study showed that RIZ1, together with another two HMTs (ESET, EuHMTase) functions as an inhibitory gatekeeper, contributing to prevent recruitment of unligaded ER-mediated and constitutive activation, and dictating LSD1 dependency for liganded ER-regulated activation by opposing the functions of the three HMTs (Garcia-Bassets I et al, 2007),

indicating RIZ1 has dual functions in regulating NR activity, dependent or independent its enzyme activity. Methyltransferases MLL2/3/4, targeting H3K4, which were linked to transactivation, were shown to serve as NR coactivators (Mo R et al, 2006; Lee S et al, 2006). Both PRMT1 and CARM1 function as a secondary coactivator for NR through its association with P160 coactivators, meaning that PRMT1/CARM1 enhance transcriptional activation by nuclear receptors dependent on the presence of P160 coactivators (Wang H et al, 2001). Another member of PRMT family proteins, PRMT2, enhances NRs mediated transactivation through its direct interaction with NRs (Qi C et al, 2002; Meyer R et al, 2007). Even though a different mechanism was undertaken by the three PRMTs in regulating NR activity, their methyltransferase activity is indispensable for the coactivator function.

Enzyme	Substrate	NR	Coregulator	Reference
Methylases				
SUV39H1	H3K9	TR	corepressor	Li J et al, 2002
G9a	H3K9	AR, ER	coactivator	Lee DY et al, 2006
SETDB1	H3K9	PPARγ	corepressor	Takada I et al, 2007
RIZ1	H3K9	ERα	coactivator	Carling T et al, 2004
RIZ1,ESET,EuHMTase	H3K9	ERα	corepressor	Garcia-Bassets I et al, 2007
MLL2	H3K4	ERα	coactivator	Mo R et al, 2006
MLL3/4	H3K4	RAR	coactivator	Lee S et al, 2006
EZH2	H3K27	ER	corepressor	Hwang C et al, 2008
NSD1	H3K36	TR, RAR	bifunctional	Huang N et al, 1998
PRMT1	H4R3	AR	coactivator	Wang H et al, 2001
PRMT2	H4, H3R8	TR, ER, AR	coactivator	Qi C et al, 2002; Meyer R et al, 2007
CARM1	H3R17,26	TR, ER, AR	coactivator	Chen D et al, 1999
PRDM16	unknown	PPARγ	coactivator	Seale P et al, 2008
Demethylases				
LSD1	H3K9me1/2	AR	coactivator	Metzger E et al, 2005
		ERα	coactivator	Garcia-Bassets I et al, 2007
	H3K4me1/2	TLX	corepressor	Yokoyama A et al, 2008
JHDM2A	H3K9me1/2	AR	coactivator	Yamane K et al, 2006
JMJD2C	H3K9me2/3	AR	coactivator	Wissmann M et al, 2007
JMJD2A/2D	H3K9me3	AR	coactivator	Shin S et al, 2007

Table 1 Summary of HMTs and HDMs that are known NR coregulators.

SUV39H1: Suppressor of variegation 3-9 homolog 1; SETDB1: SET domain, bifurcated 1; RIZ1: Retinoblastoma protein-interacting zinc finger; NSD1: Nuclear receptor binding SET domain protein 1; PDAM16: PRD1-BF1-RIZ1 homologous domain containing 16; JMJD2A: JmjC domain-containing histone demethylase 2A.

Another interesting example of histone modifier in regulating NR activity is LSD1. The ability of LSD1 to demethylate H3K4 suggested a role of LSD1 in transcriptional repression, in support of this idea, LSD1 has been shown to repress TLX (an orphan receptor) mediated gene expression through the demethylation of H3K4me1/2 (Yokoyama A et al, 2008), however, LSD1 was also shown to function as a

coactivator for AR/ER/ERR by demethylating H3K9 (Metzger et al, 2005; Garcia-Bassets I et al, 2007; Carnesecchi J et al, 2017), indicating that the complexes in which LSD1 resides tightly coordinate its gene regulatory functions and also influence its substrate specificity for histones. From the accumulating evidences, it seems likely that histone methylation/demethylation underlies ligand-dependent transcriptional control by NRs.

Other histone-modifying enzymes have been documented regulating NR activity by affecting chromatin structures. For example, a histone H2A deubiquitinase specific for monoubiquitinated H2A (H2Aub), has been shown to participate in transcriptional regulation events in androgen receptor-dependent gene activation by coordinating histone acetylation and deubiquitination, and destabilizing the association of linker histone H1 with nucleosomes (Zhu P et al, 2007). Protein-kinase-C-related kinase 1 (PRK1) and Mitogen- and stress-activated protein kinase-1 (MSK1) have been shown to serve as coactivators for AR and PR respectively by deposing phosphorylation marks on Histone3 (Metzger E et al, 2008; Vicent GP et al, 2006). A recent study also shows that phosphorylation of histone H3T6 by PKCbeta(I) contributes to AR mediated gene activation by cross-talking with LSD1 mediated H3 demethylation (Metzger E et al, 2010). Moreover, with the characterization of new type of histone modifications, it is believable that additional coregulators with precise roles in chromatin reorganization are expected to be defined in the near future.

1.3.2.3 DNA methylation Modifiers are NR Co-regulators.

Methylation of DNA occurs on the cytosine bases, primary resides within CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs). Enzymes implicated in maintenance and de novo DNA methylation are, respectively, DNA methyltransferase1 (DNMT1), and DNMT3a and DNMT3b (Niehrs C et al, 2009). DNA hypermethylation is usually associated with silent condensed chromatin state, while hypomethylation is more related to active chromatin state. The mechanisms whereby DNA methylation represses genes has been proposed to occur through either directly preventing transcription factor binding or creating a binding site for methyl-binding proteins (which I will discuss in detail later). DNMTs physically bind to HMTs and HDACs, also providing a mechanistic link between transcriptional repression via DNA hypermethylation and histone methylation and deacetylation (Fuks F et al, 2000; Fuks F et al, 2003; Estève PO et al, 2006; Viré E et al, 2006).

Studies using chromatin immunoprecipitation (ChIP) demonstrated that, liganded ERα induces an ordered and cyclical recruitment of coactivator complexes (which I will discuss in detail later) (Metivier R et al, 2003), meanwhile, an unexpected cyclic variation of CpG methylation was also observed: methylation of CpGs, which occurs at the end of each transcriptionally productive cycle is correlated with the presence of MeCP2, SWI/SNF, DNMT1 and DNMT3a/b on the pS2 (an ERα target gene) promoter, while demethylation of pS2 promoter CpGs involves the coordinate recruitment of DNMT3a/b, p68, TDG and BER proteins (Metivier R et al, 2008). This DNMTs-dependent variation of DNA methylation provides an additional level of epigenetic regulation to NR-mediated target gene expression.

Whereas the mechanisms of DNA methylation have been established clearly, the processes that lead to active DNA demethylation remains an area of active investigation. Mounting evidence now offers a model for active DNA demethylation: Ten-eleven Translocation (TET) enzymes catalyze iterative oxidation of methylated cytosine, an unmodified cytosine is generated with the help of TDG/BER mediated base excision repair (Tahiliani et al, 2009; Ito et al., 2011; He et al., 2011). TDG was shown to be a coregulator for NR through its potentiation of estrogen receptor α (ERα) activity. The coactivator activity did not require a functional TDG catalytic domain (Chen D et al, 2003), it may be due to at least in part its interaction with transcriptional coactivators as evidenced by the interaction of TDG and CBP/p300 and SRC1 (Tini M et al, 2002; Lucey MJ et al, 2005). Another study have shown that ERα interaction with TDG increases the glycosylase activity of the enzyme (Jost, JP et al, 2002), together with the identified role of TDG in active DNA demethylation process, it is highly possible that TDG could act to promote and/or help to maintain the demethylated status of CpG dinucleotides in promoters of estrogen-responsive genes.

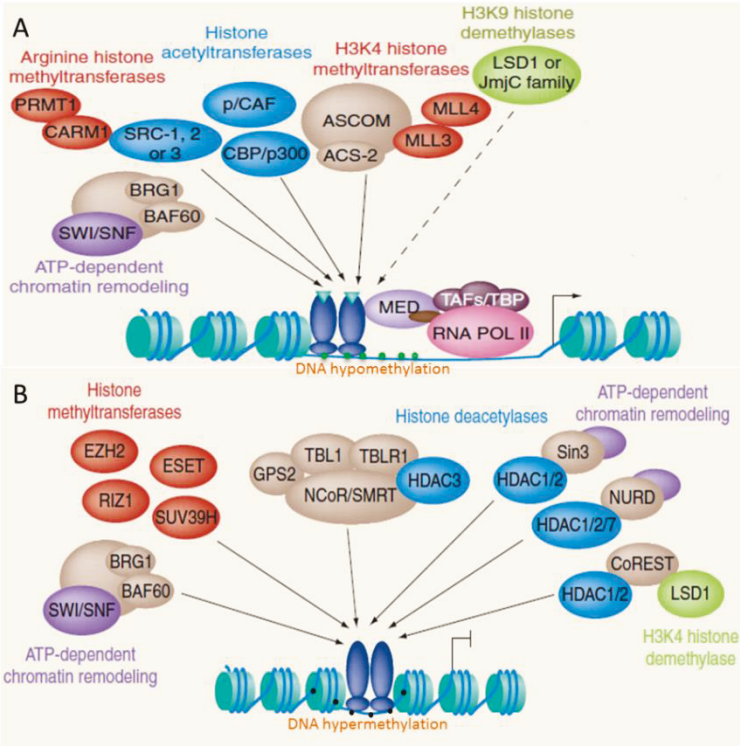


Figure 4 Nuclear Receptor Coregulators and the Respective Epigenetic Roles (modified from Green CD et al, 2011).

(A)Ligand-induced active transcription of nuclear receptor target genes requires either simultaneous or ordered recruitment of cofactors capable of modifying histones and cofactors capable of maintaining DNA hypomethylation state. Histone modifications mediated by coactivators include acetylation (SRC1, SRC2 or SRC3, p/CAF, CBP/p300), methylation (PRMT1, CARM1, MLL3 and MLL4), demethylation (LSD1, JmjC family) and ATP-dependent chromatin remodeling (SWI/SNF). (B) The absence of ligand allows nuclear receptors to interact with corepressors capable of depositing repressive epigenetic marks on histone or DNA. Corepressors function as multisubunit complexes

containing histone modifying activities such as ATP-dependent chromatin remodeling (SWI/SNF), methylation (EZH2, ESET, RIZ1 and SUV39H), demethylation (LSD1) and histone deacetylation (HDACs). The deacetylase HDAC3 is primarily associated with both the NCoR and SMRT complexes and their components GPS2, TBL1 and TBLR1. SIN3A, NURD and CoREST also deacetylate histones through HDAC1/2/7.

Another key protein involved in active DNA demethylation is TET family proteins (which I will discuss in detail in the manuscript of my first project), upon the identification of their enzyme activity, TET proteins become focus of substantial studies. Although reports have demonstrated a role of TETs with transcriptional regulation, limited studies reported TETs involving in NR mediated transcription regulation (including PPARγ and GR). Since TET proteins were shown to have a dual role in transcriptional regulation, dependent or independent on its enzymatic activity towards methylated DNA (Williams K et al, 2011). Further studies related to the modes of action of TET proteins will shed light on how these versatile proteins could modulate NR activity.

1.3.3 Ordered and Dynamic Recruitment of Coregulators

As the identified number of potential coregulators clearly exceeds the capacity for direct interaction with a single receptor at the same time, one most plausible hypothesis is that different protein complexes can act either sequentially, combinatorially, or in parallel, to reorganize chromatin templates and to modify and recruit basal transcriptional machinery.

The most detailed ChIP-based analysis of the dynamic cofactors recruitment has been obtained for ERα-mediated gene expression on pS2/TFF1, cyclin D1, cathepsin D (CATD) and c-Myc promoters(Shang et al, 2000; Reid et al, 2003; Métivier et al, 2003 ; Liu & Bagchi, 2004; Park et al, 2005). These studies indicate that the recruitment of coregulators follows a precise timed and ordered pattern. Take the dynamic interaction between the pS2 promoter and liganded ERα and its cofactors as an example, three types of cycling occur in the presence of oestradiol (E2): the cyclic transcriptional event begins with an initial, transcriptionally unproductive recruitment of ER, Brg1, PRMT1, P300/TIP60 and TAFs (TBP associated factors), followed by two alternating, transcriptionally productive cycles recruiting p68 RNA helicase, HMTs, HATs, TAFs, mediators, polII, elongator and SWI/SNF (Métivier et al, 2003). Sequential chromatin immunoprecipitations (Re-ChIP), which detect the simultaneous presence of two proteins on promoter, have identified different ERα-containing complexes in the presence of E2. Interestingly, Re-ChIP data also point to alternative recruitments for functionally redundant proteins, such as p300, CBP or Tip60; PRMT1 or CARM1; SRC1 or SRC3; Brg1 or Brm1 (Métivier et al, 2003). The dynamic recruitment of different coregulator complexes is associated with an equally dynamic binding of ERα itself to the promoter. At the end of each cycle, a cyclic inactivation of the promoter was also observed. Proteins involved in this promoter "clearance" includes SWI/SNF complex associated with HDACs and NURD complex (Métivier et al, 2003). This cyclic recruitment of SWI/SNF during clearance phase indicates a role of chromatin remodelling factors in resetting the permissiveness of the promoter for transcription. Furthermore, a subset of proteasome, termed APIS, is also involved in the clearance of ER from the promoter (Reid et al, 2003). Moreover, a similar coordinated recruitment of corepressor complexes was also observed for transcriptional repression mediated by 4-hydroxy-tamoxifen (4-OHT)-bound ERα. Liu & Bagchi et al. has shown that the recruitment of NCoR-TBL1-HDAC3 and the chromatin-remodelling

complexes NURD (nucleosome remodelling and histone deacetylation) occurs in a sequential manner(Liu & Bagchi et al, 2004).

Collectively, nuclear receptors carry out their many different transcriptional functions through the recruitment of a host of positive and negative regulatory proteins, the unexpectedly large array of coregulatory proteins bearing different enzymatic activities converges at promoters, defining what could be considered a “coregulator code”. The transcriptional coregulation of these coregulators was always linked to epigenetic modifications, such as chromatin remodeling, histone modifications and DNA methylation. Moreover, NRs could be recruited to cognate hormone response elements in a cyclic manner in the presence of ligands. In addition, diverse cofactor complexes as well as basal transcription factors have been recruited to the promoter element in coordination with cyclic loading of NRs.

Even though different cofactors harbor different regulatory functions towards NRs, it is should be kept in mind that it is not always possible to assign a functional outcome on the basis of the recruitment of a specific cofactor. Summarized from the cofactors we discussed above, some cofactors, such as, chromatin remodeling complex, which have been associated with the regulation of both gene activation and gene repression. Moreover, the ligand-dependent recruitment of some cofactors, such as LCoR and RIP40, are not associated with ligand induced transactivation. Therefore, it is important to consider that the role of a given coregulatory protein can be really context dependent.

Furthermore, new coregulators are continually discovered, including factors that were not expected to serve such functions, as exemplified by the RNA transcript for the steroid-receptor-RNA activator-1 (SRA1) coactivator (Lanz RB et al, 1999). Additionally, more and more NR interacting proteins, which have the ability to regulate NR activity through different mechanism that doesn’t fit in the conventional picture of “coregulator code”, were identified (which I will discuss in details for TR interacting proteins). This indicates that transcriptional regulation of NRs cannot be considered as an independent, chromatin-based process, but rather should be considered as coupled to many other cellular events that are carried out by several distinct groups of factors.

2 Thyroid Hormone Receptors

2.1 Thyroid Hormones

Thyroid hormone (TH) exerts its actions on virtually all tissues of mammals. The formation of the thyroid hormones depends on an exogenous supply of iodide. The thyroid gland is the only tissue of the body able to accumulate iodine in large quantities and incorporate it into hormones. Iodine absorbed from the gastrointestinal system immediately diffuses in extracellular fluid. T3 and T4 hormones are fundamentally formed by the addition of iodine to tyrosine amino acids. T4, which has a longer half-life, is the major hormone secreted by the thyroid, there is much higher levels of T4 than T3 in the circulation. Though the most synthesized hormone in thyroid gland is T4, the most efficient one is T3, the enzymatic conversion of T4 to T3 is an obligate step in the physiologic action of thyroid hormones. T4 is converted to the active T3 by type I and type II deiodinases (also termed as D1, D2). Type III deiodinase (D3) inactivates T4 to 3,3',5'-triiodothyronine (rT3) and T3 to 3,3'-diiodothyronine (T2) (Figure.5A).

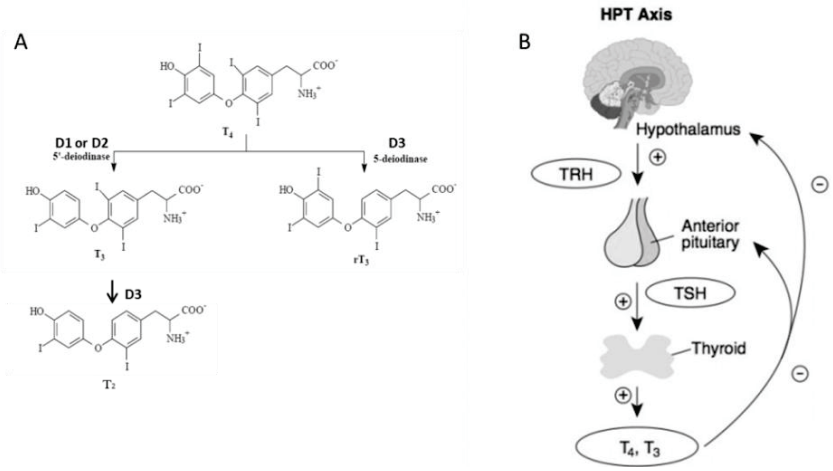


Figure 5 Chemical Structure and Hypothalamus-Pituitary-Thyroid Regulation of Thyroid Hormones.

(A) The active form of thyroid hormone, T3 (3,5,3',-triiodo-L-thyronine), is generated from its precursor T4 (3,5,3',5'-tetraiodo-L-thyronine,T4), catalyzed by a group of deiodinating enzymes. The deiodinases are classified into three different subtypes: type I, II and III (also termed as D1, D2 and D3). Type I and type II deiodinases catalyze the deiodination process from T4 to T3, while Type III deiodinase serves to inactive TH by catalyzing the deiodination process from T4 to rT3 (3,3',5'-triiodothyronine) or from T3 to T2 (3,3'-diiodothyronine). (B) The circulating level of thyroid hormone is strictly controlled by the hypothalamus, pituitary and thyroid gland. Once the hypothalamus sense the low circulating level of TH, thyrotropin-releasing hormone (TRH) will be secreted, which stimulate pituitary to release thyroid-stimulating hormone (TSH), TSH then stimulates thyroid gland to synthesis more TH. In reverse, a high circulating level of TH will in turn to inhibit the releasing of TRH and TSH by hypothalamus and pituitary separately.

Synthesis and secretions of thyroid hormones need to be kept at a certain level. In this respect, the most important mechanism in controlling the synthesis and secretion of thyroid hormones is the hypothalamus-pituitary-thyroid axis (Figure.5B). The regulation of thyroid hormone levels by hypothalamus-pituitary-thyroid (HPT) axis begins with thyrotropin-releasing hormone (TRH) synthesis and secretion by hypothalamus after sensing low circulating levels of thyroid hormone, the TRH stimulates the pituitary to produce thyroid-stimulating hormone (TSH), TSH is then transferred by blood and stimulates the thyroid gland, and thus, thyroid hormone synthesis and secretion begins (Chiamolera MI et al 2009). Thyroid hormone exerts negative feedback control over the hypothalamus as well as pituitary, thus controlling the release of both TRH from hypothalamus and TSH from pituitary gland.

The active sites of the deiodinases are located intracellularly, and the genomic action of TH (Thyroid hormone) takes place inside the cells, TH metabolism and action require transport of the hormone from extracellular compartments across the plasma membrane. Based on their lipophilic nature, it was assumed previously that translocation of iodothyronines across the lipid bilayer of cell membranes occurred by diffusion. However, accumulating evidences show clearly that TH traverses the cell membrane mainly through transporters (Hennemann G et al, 2001), which adds another layer of regulation towards intracellular availability of THs.

Collectively, the biological activity of thyroid hormone in target cells is determined by the intracellular availability of T3, which depends on a number of factors, including (i) the concentrations of T3 and its precursor T4 in the circulation; (ii) the activities of the iodothyronine deiodinases D1 and D2 that activate thyroid hormone by converting T4 to T3 as well as the activity of deiodinase D3 that inactivate thyroid hormone by catalyzing the formation of 3,3',5'-triiodothyronine (rT3) from T4 and 3,3'-diiodothyronine (T2) from T3; and (iii) TH transport from extracellular compartments(i.e. bloodstream) across the plasma membrane by groups of transporters; (iV) the availability of thyroid hormone receptor and its cofactors (which will be detailed discussed later).

2.2 Thyroid Hormone Receptor Functional Domains

The genomic actions of T3 are mediated by thyroid hormone receptors (TRs). TRs are members of the nuclear receptor superfamily and function as T3-inducible transcription factors. Two TR genes (*THRA* and *THRB*) encode four main TR isoforms: TRα1, β1, β2 and β3 (which I will discuss later in multiforms of TR part). Similar to other members of the nuclear receptor superfamily, TRs consist of three function domains: the N-terminal A/B domain, a central DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD), a hinge region containing the nuclear localization signal located in between the DBD and LBD domains (Figure.6). It should be noted that each of these domains may have multiple functions, and thus their names may only reflect the first function ascribed to them.

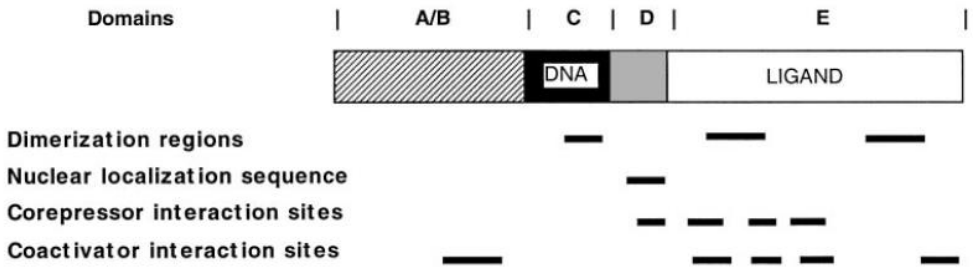


Figure 6 General domains and functional subregions of TRs (Yen PM et al, 2001).
TRs have similar domain organization as found in other NR, the N terminal A/B domain, the central DNA binding domain, the hinge region and the C terminal ligand binding domain. The underlined subregions are involved in different functions including dimerization, nuclear localization and co-regulator interaction.

2.2.1 N-terminal A/B domain

The N-terminal regions of TRs are highly variable in both amino acid sequence and size among different TR isoforms. The role(s) of this amino-terminal domain is poorly understood. Studies of steroid hormone receptors have suggested that it contains an independent constitutive activation function (Derek N. Lavery et al, 2005). However, the role of the amino-terminal domain of TR in transcriptional activation is still controversial. Some studies have shown that deletion of the amino-terminal domain of TRβ1 had no effect on T3-dependent transcriptional activation (YEN PM et al, 1995), on the other hand, other studies indicate the existence of ligand independent activation function domain (AF-1) domain at the amino-terminal of TR (Wilkinson JR et al, 1997; Wu Y et al, 2001). It is possible that differences in species, cell

types, TREs (TH Response Elements) may account for these different observations. Notably, TRβ2, compared to other TR isoforms, has a longer N-terminal A/B domain. This unique N-terminus of TRβ2 isoform permits additional ligand independent interaction with coactivators that are not allowed by the N-terminus of other TR isoforms, contributing to the hormone-independent transcriptional activity of TRβ2 (Lee S et al, 2011). Additionally, the amino-terminal domain of TRs also may influence the conformation of the DBD and the repertoire of TREs to which it can bind (HADZIC E et al, 1998).

2.2.2 DNA-Binding Domain

DNA-binding domain, located in the central of TR, is highly conserved in sequence and structure. It contains two zinc fingers, each composed of four cysteines coordinated with a zinc ion (Figure.7). Zinc fingers are critical in mediating DNA binding, since deletion of zinc fingers or amino acid substitution of the cysteine residues abrogates DNA-binding and transcriptional activity of TR (NAGAYA T et al, 1992; YEN PM et al, 1995). Within the DBD, there is a "P box" and an "A box" region, which are responsible for mediating its interaction with the major and minor groove of TRE (TH response element). The "A box" is located downstream of the second zinc finger, and the "P box" is located within the first zinc finger, which has been shown to be important in sequence-specific recognition of hormone response elements by different members of the nuclear hormone superfamily including TR (Pawlak M et al, 2012). A "D box" located within the second zinc finger was shown to be involved in mediating homodimer formation and contributing, to a much lesser extent, to heterodimer stabilization. A "T box" region located downstream of the second zinc finger was also shown to be involved in forming the dimerization interface of TR (Bain DL et al, 2007).

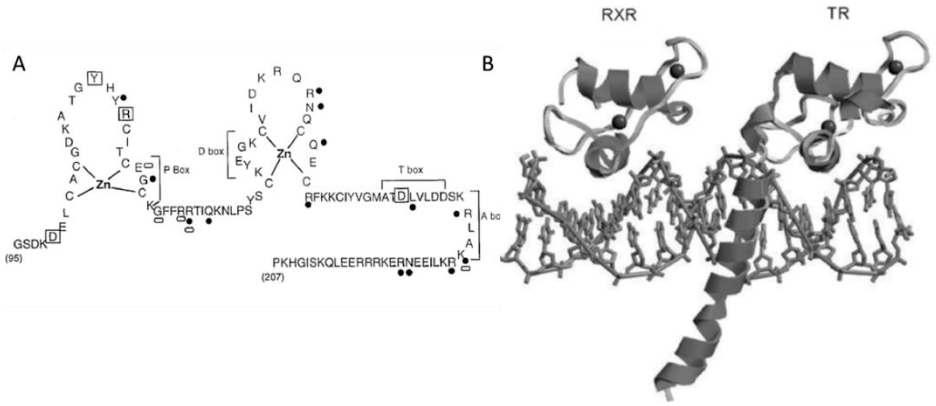


Figure 7 Structure of DNA binding domain of TR (Yen PM et al, 2001; Bain DL et al, 2007).
(A) Schematic drawing of the two zinc fingers of human TRβ and the various subregions (including the "P box", the "A box", the "D box" and the "T box"). Squares, TR/RXR heterodimerization contacts; ovals, direct base contacts; solid circles, direct phosphate contacts. (B)Three dimensional structure of DNA binding domain of TR heterodimerized with RXR-DBD on DNA (DR4, direct repeat of two half sites separated by 4 nucleotides). TR/RXR heterodimers bind to DR4 with a 5' to 3' polarity with TR in the downstream position.

Additionally, as discussed below, TRs can heterodimerize with RXRs and can bind to TREs that are arranged as direct repeats separated by a four nucleotide gap (DR4). The tandem arrangement of the half sites in DR4 TRE imposes a head-to-tail orientation of the DBDs of RXR and TR. Moreover, although

the two half site share consensus sequences, the RXR DBD occupies the upstream DNA element and the TR DBD occupies the downstream DNA half-site (Figure.7) (Bain DL et al, 2007).

2.2.3 Ligand-Binding Domain

The multifunctional ligand binding domain (LBD) is not only responsible for TH binding, but also plays critical roles for dimerization, transactivation, and basal repression by unliganded TR. The solutions of the crystal structures of the liganded TRα, unliganded RXRα have greatly aided our understanding of its role on these functions and the attendant conformational changes that occur when T3 binds to the receptor. The LBD consists of a single structural domain packed in three layers, composed of 12 α-helices and four short β-strands forming a mixed β-sheet (Figure.8) (Wagner RL et al, 2001). TH is buried deeply within a hydrophobic pocket in the LBD formed by almost the entire LBD. Particularly, the carboxy-terminal most region (Helix 12) contributes its hydrophobic surface as part of the ligand binding cavity. Moreover, the hydrophobic interfaces formed by discontinuous helices have also been proposed to be involved in TR homo- and heterodimerization (Wagner RL et al, 2001).

Although the crystal structure of unliganded TR has yet to be solved, the crystal structures of unliganded RXR and ER show that helix 12 projects into the solvent (Figure.8) (Huang P et al, 2010). Thus it is likely that, similar to other NRs, helix 12 of TR undergoes major conformational changes upon ligand binding, from a more open conformation to a closed one. The open conformation favors TR interaction with co-repressors interaction while the ligand induced helix 12 re-position favors TR recruitment of coactivators (Lonard DM et al, 2007). The helix 12 is the major architecture associated with C terminal activation domain, which is implicated in hormone dependent transcriptional activation. Accordingly, mutations of key residues within this domain decrease or abolish ligand-dependent activation (Quignodon L et al, 2007; Bochukova E, et al, 2012).

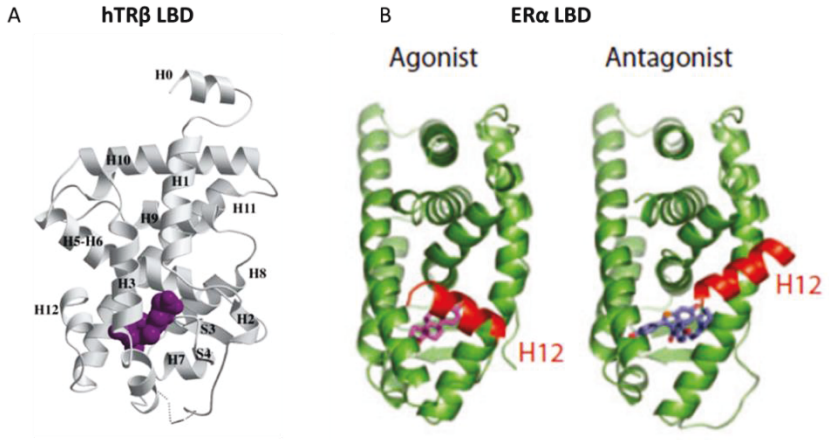


Figure 8 Structure of ligand binding domain of TR and ER (Wagner RL et al, 2001; Huang P et al, 2010)
(A) Three dimensional structure of ligand binding domain of hTRβ. LBD consists of 12 α-helices, H1-H12, and four short β-strands. (B) H12 geometry and activation function-2 (AF-2) function is ligand dependent. Erα agonists, such as the natural ligand 17β-estradiol (E2) treatment causes a conformation change in the LBD, particularly in H12 (red), from a more open conformation to a closed one.

2.3 Multiple Forms of Thyroid Hormone Receptors

TRs are derived from two genes (*THRA* and *THRB*) located on two different chromosomes. The *THRA* gene, located on chromosome 17, encodes one T3-binding TRα1 and two splicing variants (TRα2 and TRα3). These TRα1 variants, which are different from TRα1 in C-terminal region, have no T3-binding activity (Mitsuhashi T et al, 1988). Internal promoter gives rise to TRΔα1 and TRΔα2 that lack amino-terminal A/B and DNA domains but retain most of the T3-binding domain (Plateroti M et al, 2001). The P43 mitochondrial protein has been proposed to be a TRα1 mRNA translation product initiated at an AUG codon located downstream of the first consensus initiation codon and to mediate the mitochondrial response to T3 (Casas F et al, 1999) (Figure.9). More recently, a 30kDa TR isoform, corresponding to the isolated ligand binding domain, has been identified. It is an isoform localized at the plasma membrane and was shown to be involved in mediating some non-genomic effect of TH (Kalyanaraman H et al, 2014).

The *THRB* gene is located on chromosome 3, it encodes three T3-binding TRβ isoforms (β1, β2, and β3). These TRβ isoforms share high sequence homology in the DNA and ligand binding domains but differ in the length and amino acid sequences in the N-terminal A/B domain. Truncated TRs, transcribed from an internal promoter leads to the TRΔβ3 that lacks the amino A/B and DNA-binding domains but retains T3-binding activity (Williams GR et al, 2000) (Figure.9).

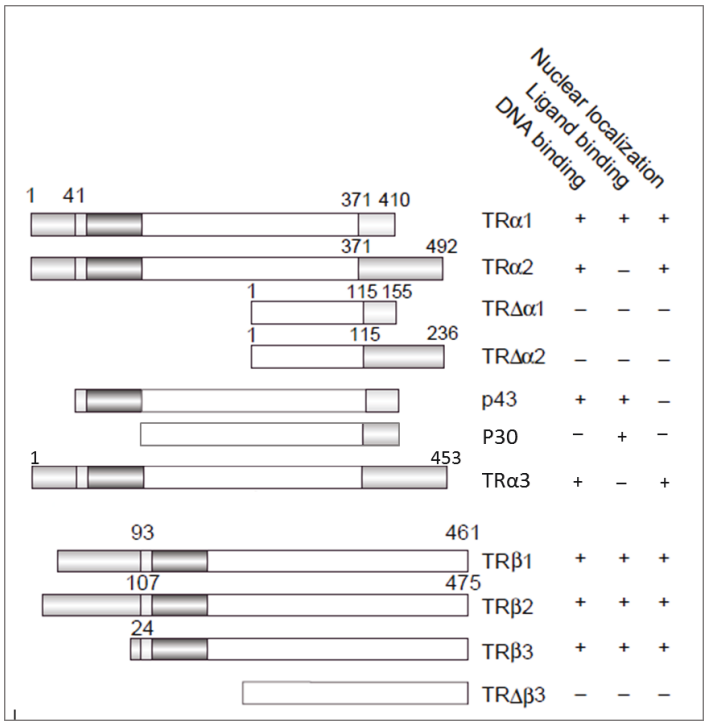


Figure 9 Schematic representation of TR isoforms (Flamant F, Samarut J. et al, 2003).

TRs are encoded by the *THRA* and *THRB* genes located on different chromosomes. Alternative splicing and the usage of internal ATG of primary transcripts give rise to different thyroid hormone binding isoforms.TRα1, TRβ1, TRβ2 and TRβ3 are four main isoforms which retain both DNA binding and ligand binding abilities, while other isoforms show different ligand and DNA binding abilities and/or nuclear localizations.

TR isoforms exerts a tissue-dependent and developmentally regulated expression pattern. TRα1 and TRα2 are expressed at the highest levels in the brain; at lower levels in the kidneys, skeletal muscle, lungs, heart, testes, and liver; TRβ1 is expressed predominantly in the kidneys, liver, brain, heart, and thyroid; TRβ2 is mainly expressed in the brain, retina, and inner ears; and TRβ3 is predominantly expressed in the kidneys, liver, and lungs (Williams GR et al, 2000). TRα is constitutively expressed at embryonic development, and TRβ is expressed toward the later stage of development (Darras VM et al, 2011).

2.3.1 Isoform-dependent Functions of TRs

The molecular diversity of TRs raises the question as to whether the TR isoforms have distinct functions or simply serve a redundant role for each other. The tissue-dependent and developmentally regulated differential expression of TR isoforms suggests that TRs may mediate subtype-specific functions. Phenotypes got from TRα or TRβ single or double knockout mice provide in vivo evidence to indicate that the TR isoforms can have both subtype-specific and overlapping functional roles. TRα1 KO mice have a lower heart rate, abnormal heart contractility, and decreased body temperature (Wikstrom L et al, 1998). In contrast, mice in which the TRβ gene is selectively inactivated have a mild dysfunction of the pituitary-thyroid axis and a deficit in auditory function and eye development (Jones I et al, 2007). When both TRα and TRβ genes are inactivated, an array of phenotypes are detected, including severe dysfunction of the pituitary-thyroid axis and retarded growth, which are not found in the single receptor-deficient mice (Gauthier, K. et al. (1999). These findings indicate that TRα1 and TRβ can substitute for each other to mediate some actions of T3 and also mediate isoform-specific functions.

A concerted effort has also been made to understand the possible function of the non-ligand-binding TR isoforms. TRα2, which is a poor RXR heterodimer partner, still retain its DNA binding ability, but cannot bind ligand, while TRΔα1, TRΔα2 and TRΔβ3 lost both DNA binding and ligand binding abilities. Studies showed that these four non-ligand binding isoforms can act as TR antagonists in transfected cells (Chassande O et al,1997 ; Williams GR, 2000). However the mechanisms underline the antagonist effects are till elusive. Deleting the TRΔα1and TRΔα2 isoforms, while keeping TRα1 and TRα2, entails increased T3 sensitivity, confirming that these isoforms can attenuate the intestinal mucosal response to T3. The striking phenotypic difference observed between TRα0/0 and TRα-/- mice: the lethality and severe impairment of the intestinal maturation in TRα-/- mice are rescued in TRα0/0 animals, indicating an important function of TRΔα protein isoforms in the absence of nuclear T3 binding isoforms. While how TRΔα1 and TRΔα2 exert their detrimental effect in a TRα-/- context is unclear (Gauthier K et al, 2001).

The TR isoform-dependent phenotypes prompted the question as to whether TR isoforms specifically regulate certain target genes. Recent studies provided compelling evidence that TRα1 and TRβ1 are intrinsically different in transcriptional regulation. Winter, H. et al demonstrated that in inner ear hair cells that express both TRα1 and TRβ1, KCNQ4 is specifically regulated by TRα1, whereas prestin can be activated only by TRβ1(Winter, H. et al, 2006). Gauthier K et al also found that in adipocytes, the promoter of the ChREBP gene binds both TRα1 and TRβ1, as shown by chromatin immune-precipitation,

but can be activated only by liganded TRβ1(Gauthier K et al, 2010). Recently the study on TR isoform specificity has been extended to a genome-wide scale. The HepG2 hepatoma cells that express either TRα1 or TRβ1 have been generated and used for study. While most TR target genes can be activated by either TRα1 or TRβ1, a fraction of genes display a marked preference for one of the isoforms (Chan IH et al, 2009). Similarly, in the C17.2 neural stem cell lines that stably express either TRα1 or TRβ1 at similar levels, T3 treatment leads to the activation of two clearly different sets of targets that are only partially overlapping, and this marked preference for TRα1 or TRβ1 is not correlated with differential chromatin binding of the receptors (Chatonnet F et al, 2013).

2.4 Modes of Action

2.4.1 Thyroid Hormone Response Element

TRs are DNA-binding transcription factors that recognize specific DNA sequences, called thyroid hormone response element (TRE), on the promoters of T3 target genes. TREs contain a core consensus sequence of the hexanucleotide "half-site" (A/G)GGT(C/A/G)A. The TRE half sites always exist in pairs and can be arranged in different orientations. The spacing between the two half-sites also varies, depending on the orientation of the half-sites. Analyses of the TREs in the promoters of many T3 target genes gave rises to three different TREs: everted repeats separated by six nucleotides(ER+6); direct repeat with a spacing of four nucleotides (DR+4); and inverted repeat with no space (IR+0) (Figure.10). Although TR can bind DNA as homodimers in vitro, they are thought to mainly transactivate target genes expression after heterodimerization with another member of the nuclear receptor family, the retinoid X receptor (RXR). According to structure analysis, RXR recognizes the 5' half-site (5'AGGTCA) and TR recognizes the 3' half-site (3'AGGNCA).

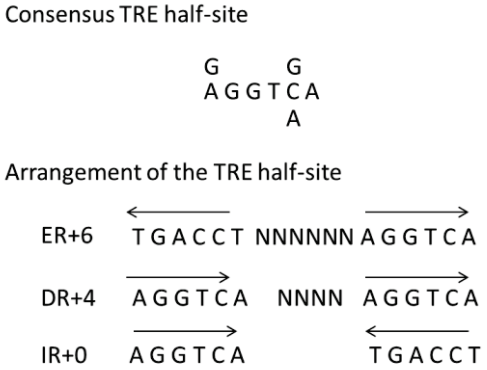


Figure 10 The DNA consensus sequence and the arrangement of the TRE half-site binding motifs.
The binding sites of TR on DNA are termed as thyroid hormone response element (TRE). TREs consist of two "half site" (core consensus sequence of the hexanucleotide (A/G)GGT(C/A/G)A) arranged in different orientations and separated by different length of nucleotides. ER+6 represents everted repeat separated with 6 nucleotides; DR+4 represents direct repeat separated with 4 nucleotides; IR+0 represents inverted repeat with no space.

Chromatin occupancy of TR was assayed at genome wide scale (ChipSeq) to identify all TR binding sites (the TR "cistrome") present next to TR responsive genes (Chatonnet F et al, 2013; Ramadoss P et al,

2014; Grontved L et al, 2015). ChipSeq analyses indicate that DR4 elements are predominant over other types of T3 response elements, which strongly suggests that the main, if not exclusive, mode of T3-induced transactivation is mediated by TR/RXR heterodimers. Additionally, around half of the TR binding sites have no signature of the consensus motif, suggesting the existence of alternate mode of transactivation, such as the cross-talk between TR and other transcription factors (Cheng SY et al, 2010).

Though informative, these ChIP-Seq data also brought new questions. For example, DR4-like elements are highly present within the genome, only a small subset of DR4 is occupied by TR. The presence of putative DR4 is thus unpredictable for TR binding and T3 regulation of nearby genes. For other nuclear receptors, such as AR and ER, the limited genomic binding is thought to result from the prerequisite binding of "pioneer" factors to prepare the binding of NR by opening the closed chromatin (Jozwik KM et al, 2012). However, there is no indication that such pioneer factors exist for TR binding. The occupancy of only a small fraction of the DR4 elements present in the genome is thus unclear for the moment. Another issue relates to the limited correlation between the occupation of a DR4 by TR and the transactivation of the neighboring gene after T3 treatment. For most of T3 response genes, a TR binding site is identified within 20Kb from the transcription start site. However, TR binding is only observed at very long distance (> 50 kb) for a number of T3-responsive genes. Such long distance regulation could be explained by chromatin looping, which brings distant sequences at vicinity (Buisine N et al, 2015). Reciprocally, a number of genes with a proximal TR binding site don't response to T3 treatment. A more detailed inspection towards the chromatin microenvironment, including the surrounding epigenetic modifications and the presence of TR coregulators, could help to understand this phenomenon.

2.4.2 Transcriptional Repression

The binding of TR with DNA is independent of ligand treatment, thus TR resides on DNA and represses the basal transcription in the absence of T3. The repressive effect is mainly due to the recruitment of corepressor proteins. The best characterized corepressor proteins include NCoR and SMRT, and as we discussed before, the mechanisms underline the repressive effects of NCoR and SMRT were better elucidated (refer to NR corepressor part).

Subsequently, other TR CoRs were identified, such as Alien (Dressel U et al, 1999) and Hairless (Potter GB et al, 2001). One mechanism by which Alien confers silencing may be based on recruitment of deacetylase activity via interaction with SIN3A (Dressel U et al, 1999). SIN3A was known to be part of a deacetylase complex (Heinzel T et al, 1997; Laherty CD et al, 1997), suggesting that Alien silence gene expression by forming mSin3A-HDAC protein complex. Accordingly, HDAC-inhibitor, trichostatin A (TSA), reduces Alien-mediated gene silencing (Dressel U et al, 1999). A further exciting mechanism of Alien-mediated silencing might occur at the level of nucleosome assembly. The nucleosome assembly protein1 (NAP1) was identified as an interaction partner for Alien. Alien was shown to enhancing NAP1-mediated nucleosome assembly on DNA, moreover the specific binding of Alien to H3 and H4 inhibits the accessibility of NAP1 to the histones H3/H4. It is therefore hypothetically possible that Alien, on one hand, promotes nucleosome assembly leading to a more compacted chromatin structure with the help

of NAP1 and, on the other hand, shifts the steady state toward prevention of histone displacement mediated by NAP1 (Eckey et al., 2007). Hairless protein (HR) functions similarly to the well-established corepressors NCoR and SMRT. It was shown that HR protein harbors multiple independent repression domains and multiple receptor interaction domains comprised of conserved hydrophobic residues (I/L-I-X-X-L/V-V) similar to those identified for NCoR and SMRT (Potter et al, 2001). Moreover, HR interacts indirectly with several HDACs, including HDACs 1, 3 and 5 (Potter et al, 2001; Potter et al, 2002). HR facilitates transcriptional repression by TR through associated HDAC activity. Consistently, HDAC inhibitor impairs HR-mediated repression (Potter et al., 2002). A JmjC domain was also recognized in the carboxyl terminus of HR protein, JmjC domains in a number of proteins function as histone demethylases (Klose et al., 2006; Tsukada et al., 2006). Recent study identified HR as a histone demethylase which can effectively demethylate monomethylated or dimethylated histone H3 lysine 9 (H3K9me1 or H3K9me2, respectively) (Liu L et al, 2014). It is therefore highly possible and interesting to investigate later that the repression function of HR may lie in providing an additional component of the histone code for regulating transcriptional activity.

Interestingly, Both Alien and hairless gene expressions were shown to be controlled by thyroid hormone in brain (Thompson et al, 1996; Tenbaum et al, 2003), suggesting that TR regulates the level of its own silencing function by regulating the expression of its corepressors.

2.4.3 From Repression to Activation

Ligand binding is the crucial molecular event that switches the function of TR from a corepressor to a coactivator. Hormone binding induces dramatic structural change of LBD domain of TR, this conformational change reduces the affinity between TR and corepressors and meanwhile enhances its affinity for coactivators. In the presence of ligand, numerous coactivators, such as P160 family proteins, CBP/p300, chromatin remodeling complex SWI/SNF, mediator complex TR-associated proteins (TRAP) complex were shown to be recruited to liganded TR and were involved in TR mediated transactivation (refer to NR coactivator part).

The identification of the nuclear corepressor exchange (NCoEx) factors uncovers an additional and unexpected layer of regulation in the molecular events that modulate the nuclear receptor switch from repression to activation. Perissi V et al reported that transcriptional activation mediated by liganded nuclear receptors (including TR, PPARγ and RAR) unexpectedly requires the actions of TBL1 and TBLR1, which serve as specific adaptors for the recruitment of the ubiquitin conjugating/19S proteasome complex, the proteasome-dependent degradation of the nuclear receptor corepressors, NCoR and SMRT, are required to fully promote the release of the corepressors in response to ligand binding (Perissi V et al, 2004).Interestingly, the NCoEx factors TBL1 and TBLR1 are intrinsic components of NCoR/SMRT corepressor complex, suggesting an important role of these cofactors in both repression and activation. This also implies that the signals to induce TR activation must activate parallel pathways to activate NCoEx factors that lead to the release of corepressors, adding a further control level, which is imposed to maintain the basal transcriptional repression mediated by unliganded TR and to avoid undesirable gene expression induced by liganded TR.

2.4.4 Gene Repression by TH/Negative Regulation by Liganded TR

Negative regulation of gene expression in response to TH (T3) is an essential part of the physiological action of thyroid hormone receptors. Specifically, several negatively regulated genes such as thyrotropin-releasing hormone (TRH) and thyrotropin (TSH) α- and β-subunits (*TSHA* and *TSHB*, respectively) are inhibited by T3 as a physiologic feedback mechanism for tightly controlling circulating thyroid hormone levels. Unlike in the case of positively regulated TR target genes, which harbor positive TREs, the nature of the negative response elements (nTREs) remain poorly defined.

Generally, in the case of negative regulation, genes are activated in the absence of T3, and hormone treatment induces rapid and strong transcriptional repression. Concerted efforts have been made to better understand the mechanisms underline this negative regulation by liganded TR. Studies showed that co-regulators modulate TR activity on negative response elements in a reciprocal manner as that on positive response elements, recruitment of CoRs by TR is associated with transcriptional stimulation and histone acetylation (Tagami T et al, 1997), while CoAs recruitments are involved in T3-dependent repression and histone deacetylation (Tagami T et al, 1999). Consistent with this working model, in the case of regulation of TSH and TRH genes by unliganded TR, overexpression of NCoR and SMRT increase the stimulation effect of unliganded TR, conversely, the stimulation effects on those genes were severely impaired by introducing a mutation into TR that abolish its interaction with co-repressors. Furthermore, SRC1 KO mice display resistance to TH (RTH) evidenced by an elevation of serum TSH levels despite an increased serum free TH levels, suggesting that SRC1 is necessary for TSH downregulation by TH (Weiss RE et al, 1999). Another study also shows that a mutated TRβ that abolish CoA recruitment failed to suppress serum TSH and pituitary *TSHB* mRNA levels (Ortiga-Carvalho TM et al, 2005).

Apart from the general involvement of co-regulatory proteins in mediating negative regulation by liganded TR, what we know more is that specific target genes might adopt distinct mechanisms for repression, and that TR-DNA binding might not always be required for gene repression by T3. A mechanism for this is suggested by studies showing that TR binding to the *TSHA* promoter appears not to be necessary for negative regulation of that gene. A two-step mechanism was proposed for this negative regulation on *TSHA* promoter: 1) the unliganded TR recruits CoRs and withdraws HDAC from the basal promoter to cause activation; 2) T3 binding leads to dissociation of HDAC from TR and thereafter binding to promoter, in parallel, the liganded TR recruits CoAs to restrict access of HATs to the basal promoter, thereby causing ligand-dependent repression (Tagami T et al, 1999).

In contrast to *TSHA*, the negative regulation of the *TSHB* gene might require TR binding to its nTRE. Studies of the *TSHB* gene show that the histone deacetylase 1 (HDAC1) constitutively binds to the nTRE and both TRβ and HDAC2 are further recruited to the nTRE of the *TSHB* gene in a ligand-dependent manner. The ligand induced recruitment of HDAC2 leads to the compaction of chromatin and subsequent transcriptional repression of TSHB gene. Consistently, inhibition of HDAC activity attenuated T3-dependent *TSHB* gene repression (Sasaki S et al, 1999).

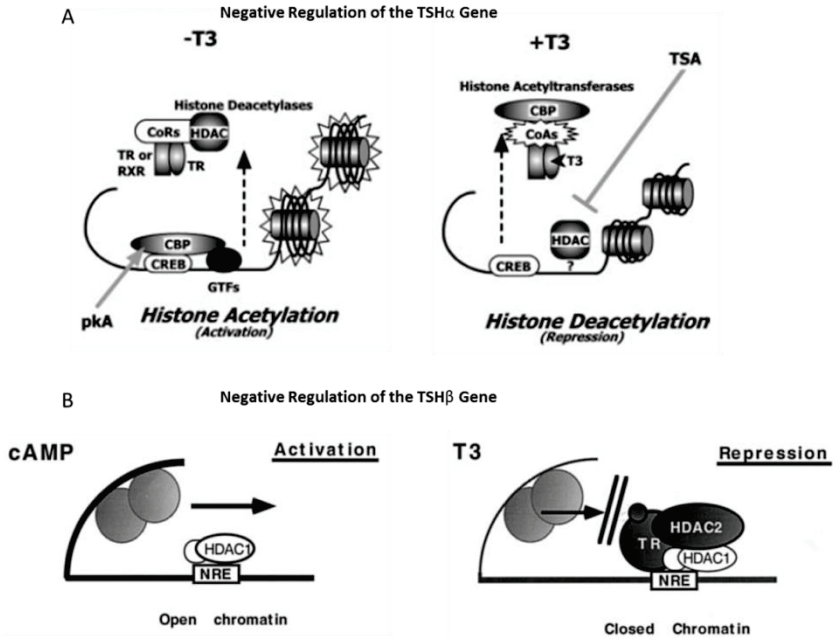


Figure 11 Working models of negative regulation of the TSH gene by T3 (Tagami T et al, 1999; Sasaki S et al, 1999).

(A) Two-step model of negative regulation of the *TSHA* gene by T3. In the absence of T3, TR binds CoRs, such as NCoR, SMRT, and SIN3A, which recruit HDAC. This TR-CoRs complex sequesters HDAC from the promoter, resulting in increased histone acetylation and an open chromatin state and therefore transcriptional activation; in the presence of T3, liganded TR recruits CoAs, which compete HATs, like CBP, away from the promoter, and the T3 induced dissociation of HDAC from TR makes HDAC available for the promoter, both of these events result in net deacetylation and transcriptional repression. GTFs, general transcription factors. (B) Model of negative regulation of the TSHβ gene by T3. HDAC1 was consistently detected on the promoter of *TSHB* gene independent of ligand treatment. In the absence of T3, cAMP activates *TSHB* gene expression by favoring an open chromatin state, even though HDAC1 is present at the promoter; T3 addition recruits TR and HDAC2 to the promoter, resulting in the closing of chromatin and transcriptional repression.

Furthermore, recent studies showed a TR isoform-specific role in mediating negative regulation of TSH genes by T3. A difference in TR isoform (TRα and TRβ) binding to the proximal promoter region of *TSHB* was observed by ChIP assays, only TRβ isoforms bind to the proximal region of *TSHB* under normal conditions, but that TRα can partially compensate in cases when TRβ is knocked down, by binding to the *TSHB* promoter (Chiamolera MI et al, 2012). These results suggest a more essential role of TRβ isoforms in regulating TSH gene expression, confering a more important and dominant role of TRβ isoforms in in the feedback regulation of HPT (hypothalamic–pituitary–thyroid) axis. The importance of TRβ isoforms in mediating T3 negative feedback on HPT axis has already been suggested by the phenotype of TR isoform specific KO mice (refer to isoform dependent functions of TR part).

Despite the working model proposed above for the negative regulation by TH, the mechanisms underline this negative regulation is still controversial. Studies suggest that TR must be bound to DNA for T3-mediated down-regulation of gene expression to occur (Shibusawa N et al, 2003). A DNA binding defective mutant of TRβ abolished transactivation on positive TREs, as well as negatively regulated promoters in TSH genes, arguing for the DNA-binding independent mechanism for thyroid hormone negative regulation in the HPT axis. The physiological significance of TRβ-DNA binding was further confirmed by the finding that TRβ-GS125 knock-in homozygous mutant (TRβGS/GS) mice displayed abnormal T3 regulation of the hypothalamic-pituitary-thyroid axis (Shibusawa N et al, 2003b). Moreover, the DNA binding independent mechanism could better explain the low frequency of proximal TR binding sites for genes that are negatively regulated by T3, based on a recent genome-wide analysis of TR functions in a neural cell line (Chatonnet F et al, 2013). The mechanisms underline TH and its receptors modulated activation of TSH expression remains to be further elucidated.

According to the proposed working model, the TH induced negative regulation is mainly associated with histone deacetylase (HDAC) activity, because the initial canonical view was that NR coregulator function was linked to histone acetylation events. With further molecular dissection of NR action, histone-modifying enzymes other than HATs and HDACs, have been documented to serve as NR coregulators (refer to NR coregulatory codes part). Further studies need to be done to explore the involvement of the emerging epigenetic modifiers, including HMTs (histone methyltransferases), chromatin remodeling complexes and DNA methylation modifiers, in regulating TR mediated negative regulation. Moreover, apart from these newly identified epigenetic modifiers, another group of NR corepressors, such as LCoR and RIP40, which are recruited to NR in the presence of hormone, have been shown to have a possible role in attenuating hormone-induced transactivation. It is also interesting to check whether these liganded TR recruited corepressors also have a function in hormone-induced target gene repression.

2.5 Multilevel Regulation of TR Activity

2.5.1 TR Interacting Proteins

Similar to other NRs, regulation of gene transcription by thyroid hormone receptors requires the recruitment of proteins characterized as coregulators. The ligand dependent exchange of corepressors for coactivators serves as the basic mechanism for switching gene repression to activation. However, in addition to the classical nuclear receptor coregulators that modulate the transcriptional activity of TR by modifying chromatin structure (as I discussed in NR coregulatory codes part), a number of TR interacting proteins, which were out of the canonical picture, were identified, indicating that the activity of TRs can also be regulated by other cellular proteins. These TR-interacting proteins are functionally diverse, the mode of interaction with TRs varies from one to the other, and the mechanisms by which these proteins modulate the functions of TR also differ.

PP32

PP32 (Phosphoprotein 32KDa) belongs to a family of evolutionarily conserved proteins known as the acidic nuclear phosphoprotein family, which is characterized by an N-terminal leucinerich repeat domain

and a C-terminal low-complexity acidic region. PP32 was first identified as a member of the inhibitor of histone acetyltransferase (INHAT) complex (Seo SB et al, 2001). Further studies revealed that PP32 blocks histone modification by binding to histone tails and sterically inhibiting acetylation. More specifically, PP32 preferentially binds to unmodified histone H3 tails (Schneider R et al, 2004; Seo SB et al, 2002).

After this discovery, pp32 has also been identified as a coregulator of NR-dependent transcription. PP32 was found associated with ERα in MCF7 cells independent of ligand treatment and to enhance ERα-ERE complex formation but to decrease ERα-mediated transcription, possibly through its ability to decrease acetylation of histones and ERα (Loven MA et al, 2004). The interaction with PP32 is not specific to ERα since thyroid receptor beta (TRβ), progesterone receptor B (PR-B), peroxisome proliferator activator receptor gamma (PPARγ) and androgen receptor (AR) were also shown to interact with PP32 (Loven MA et al, 2004; Adegbola O et al, 2005). Similar to ERα, PP32 acted as a transcriptional corepressor for TRβ in transient transfection assay. However, PP32 was shown to act as a coactivator for AR-mediated transcription and this activity is modulated by the retinoblastoma protein (Rb), another AR co-activator (S Yeh et al, 1998), by forming a Rb-PP32 complex, the underline mechanism is still elusive (Adegbola O et al, 2005). PP32 seems to be a bifunctional coregulatory protein for NR, The switching mechanisms which regulate its bifunctionality is not clear, but the coactivator or corepressor activity may depend on the specific NR reporter gene promoter and cell type.

TDG

Thymine DNA glycosylase (TDG), a key enzyme involved in DNA repair process, initiates repair of G/T and G/U mismatches in the CpG contex, commonly associated with CpG islands, by removing thymine and uracil moieties (Neddermann P et al, 1996). Recently, TDG was found to be involved in DNA demethylation process, specifically, DNA hydroxylase TETs family proteins catalyze iterative oxidation of methylated cytosine, oxidizing 5mC to 5hmC/5fC/5caC, and TDG/BER excises 5fC/5caC to regenerate unmodified cytosine (Wu H et al, 2014).

TDG was shown to be a coregulator for NR through its potentiation of estrogen receptor α (ERα) activity. A hormone modulated interaction between TDG and ERα was identified, involving the Helix 12 domain of ERα and a putative α-helical motif, similar to the LXXLL motif that mediates interaction with NR, in TDG. An interaction between TDG and other NRs, including TRα, AR, GR, PR, PPARγ, RARα, VDR and RXRα, was also observed, implying TDG as a general coregulator for NRs (Chen D et al, 2003). The coactivator activity of TDG may be due at least in part to its interaction with transcriptional coactivators as evidenced by the interaction of TDG and CBP/p300 and SRC1 (Tini M et al, 2002; Lucey MJ et al, 2005). Moreover, based on the recent identified role of TDG in DNA demethylation, it is highly possible that TDG could act to promote and/or help to maintain the demethylated status of CpG dinucleotides in promoters of estrogen-responsive genes.

CTCF

CCCTC-binding factor (CTCF) is a highly conserved zinc finger protein and is best known as a transcription factor (Filippova GN et al, 1996). It was first identified as a transcriptional repressor of c-myc oncogene (Klenova EM et al, 1993; Filippova GN et al, 1996), but was also found to act as a transcriptional activator or an insulator protein, mediating enhancer blocking by blocking the communication between enhancers and promoters (Vostrov AA et al, 2002; Bell AC et al, 1999).

CTCF performs multiple roles, and in agreement the protein shares chromatin binding sites with many other factors including thyroid hormone receptor (TR). Co-association event with TR has been described for chicken lysozyme-silencer (Baniahmad et al, 1990), the human TRE-containing genomic element 144 (awad et al, 1999) and the human c-myc genes (Lutz M et al, 2003). Although CTCF and TR interact directly, a cooperative binding to DNA could not be demonstrated (Pérez-Juste et al, 2000). For the composite CTCF/TR element located at the silencer element of lysozyme gene, both factors synergize in repression as well as in activation, the synergy of CTCF in repression mediated by TR might be achieved by its binding of SIN3A, thus recruiting histone deacetylases to the TR/CTCF-DNA complex (Lutz et al, 2000). Similarly, a composite CTCF/TR binding site is found for TRE-containing genomic element 144, this TRE is a so-called negative response element, mediating transcriptional repression of T3. This repression is critically dependent on CTCF binding next to the TRE (Awad et al, 1999), suggesting an important role of CTCF in cooperation with TR to mediate TRE-dependent transcriptional repression. Conversely, the CTCF function is also modulated by neighboring bound TR. It has been demonstrated that thyroid hormone mediates the relief of enhancer blocking even though CTCF remains bound to its binding site. Notably, the relief of enhancer blocking was accompanied by increased levels of H4 acetylation at the binding sites of CTCF and TR and at the enhancer itself, suggesting an involvement of histone modifying enzymes. The T3 induced relief of enhancer blocking is detected for the composite element of both the chicken lysozyme-silencer and the human c-myc genes (Lutz et al, 2003). This T3 induced relief of enhancer blocking might, at least partially, account for the synergy of CTCF in TR mediated activation in the presence of T3, proposing a possible role of TR in modulating gene expression indirectly by modulating the CTCF function in organizing the 3D structure of the genome.

A genome-wide searching for additional CTCF/TR composite elements was then performed (Weth et al, 2010; Chatonnet et al, 2013). No significant, but beyond random encounter, enrichment of TREs next to CTCF binding sites throughout the genome was unveiled, indicating that the co-regulation of CTCF and TR might not be a general mechanism, but on the other hand it also suggests a role of individual composite binding sites in gene regulation.

P53

The tumor suppressor p53 is a critical transcription factor involved in cell cycle regulation and tumorigenesis (Riley T et al, 2008). A physical interaction between P53 and TRβ1 was observed, the regions involved reside in the DNA binding domain of each protein and also the C terminus polymerization domain of P53 (Yap et al, 1996; Barrera-Hernandez G et al, 1998). Further functional study of this interaction revealed that P53 functions as a corepressor for TRβ1 by inhibiting its binding to

target genes; reversely, TRβ1 increase the binding of p53 to p53 DNA-binding elements, however, resulting in repression of p53-dependent transcription activation. These studies uncover a novel cross talk between TR and other transcriptional factors, herein p53, implying an important role of this cross talk in the biology of normal and cancer cells.

19S regulatory particles

The 26S proteasome degrades ubiquitinated proteins in an ATP dependent manner and regulates the turnover of short lived proteins. The proteasome holoenzyme, 26S proteasome is composed of two sub complexes: a 20S proteolytic core particle and a 19S regulatory particle, the 19S regulatory particle contains six ATPases and three non-ATPase subunits (D. Finley et al, 2009). Emerging evidence indicated that the ATPases of 19S regulatory particle play critical roles not only in the degradation of ubiquitinated proteins, but are also involved in transcriptional regulation through their association with actively transcribed promoters, facilitation of the clearance of paused elongation complex, cross-talking histone modifications, and recruitment of coactivator proteins (F. Ganzalez et al, 2002; A. Ferdous et al, 2001; E. Ezhkova et al, 2004; D. Lee et al, 2005).

A physical interaction between two ATPases of 19S regulatory particle, TBP-1 (Tat-binding protein-1) and TRIP1 (thyroid-hormone-receptor interacting protein1), and TR were identified. TRIP1 was shown to interact with the AF2 domain of TR in a ligand dependent manner, while TBP-1 interacts with the DNA binding domain of TR independent of T3 treatment (T. Ishizuka et al, 2001; Lee JW et al, 1995). Both proteins were shown to enhance TR-mediated transcription. Knocking down of TBP-1 or TRIP1 or Rpn10 (a non-ATPase subunit of 19S particle) attenuated TR-mediated transactivation, suggesting that multiple ATPase and non-ATPase components of 19SRP play crucial roles in TR-mediated activation of gene transcription. The steady state of TR protein was not altered with manipulating the levels of these subunits of 19S particle, instead, the activator functions were shown to associate with their role in promoting efficient and proper loading of liganded TR to target promoter, and thereby affecting subsequent recruitment of SRC-1 and Pol II (Satoh T et al, 2009). These studies established an involvement of multiple components of 19S regulatory particle in NR mediated gene transcription.

Basal Transcription Factors (TF)

For transcription initiation, Pol II assembles with the basal transcription factors (TF) (TFIIA, -B, -D, -E, -F, and -H) to form a functional preinitiation complex (PIC) on core promoter elements. The preinitiation complex (PIC) assembles in a specific order: the binding of TFIID to the TATA element, in a step facilitated by TFIIA, initiates PIC formation and continues with the ordered assembly of TFIIB, pol II/TFIIF, TFIIE, and TFIIH (Conaway RC et al, 1993).

Although the transcription activity of TR is mainly regulated by a host of coreulators, including the mediator complexes, which functions to communicate between TR and the basic transcription machinery, direct interactions between TR and basal transcription factors were also identified. Studies showed that unliganded TRα can repress transcription by inhibiting the formation of a functional PIC (Fondell JD et al, 1993). Consistently, a direct interaction between TRα and the basal transcription factors, herein TFIIB and TBP (TATA-binding protein, component of TFIID), were identified (Fondell JD et

al, 1993; Baniahmad A et al, 1993; Fondell JD et al, 1996). Further studies showed that unliganded TR can target TBP for transcription repression by inhibiting PIC formation (Fondell JD et al, 1996). Furthermore, a more recent study observed an interaction between TRβ and P62 (a subunit of TFIIF), p62 interacts with TRβ in a ligand dependent manner, and enhance T3 mediated transcription (Liu Y et al, 2005).Taken together, these studies suggest that TRs can interact directly with basal transcription factors and may provide an alternative pathway for TR communication with the general transcription machinery that circumvents coregulators.

BTG1

The BTG1 (B-cell translocation gene 1) gene coding sequence was isolated from a translocation break point in a case of B-cell chronic lymphocytic leukaemia (Rouault et al., 1992).

A role of BTG1 in transcription regulation was suggested by the identification of a physical interaction between BTG1 and PRMT1 (protein arginine N-methyl-transferase), a histone methyltransferase, thus controlling transcription through histone methylation. Likewise, BTG1 has been shown to interact with other proteins involved in transcriptional regulation, such as CAF-1, a component of the CCR4 transcriptional complex (Rouault et al, 1998) and Hoxb9 (homeo box B9 protein) (Prevot et al, 2000). The occurrence of two LxxLL motifs in the protein sequence suggests that BTG1 could also act as a nuclear receptor coregulator. Busson et al. identified BTG1 as a co-activator for NR through a physical interaction with nuclear receptors (TRα1, TRβ1, RARα, RXRα and PPARγ). The ligand binding domain, especially the AF2 domain, of TR is involved in its interaction with BTG1. The interaction between BTG1 and TR is T3 dependent and the ligand dependency is much stronger in the presence of NCoR, indicating a competitive interaction between NCoR and BTG1 with TR in the absence of T3. However, BTG1 coactivator activity does not involve interactions with HAT or with PRMT1, since neither HAT inhibitor nor PRMT1 overexpression influences BTG1 coactivator activity, thus the mechanism underline this positive regulation is still elusive (Busson et al, 2005).

Protein	Function	Menchanisms	reference
PP32	corepressor for ER/TR	inhibiting acetylation of histones and ERα	Loven MA et al, 2004
	coactivator for AR	forming complex with AR coactivator Rb	Adegbola O et al, 2005
TDG	coactivator for ER	interaction of TDG and CBP/p300 and SRC1	Tini M et al, 2002; Lucey MJ et al, 2005
		(possibly) through TETs/TDG mediated DNA demethylation	Wu H et al, 2014
CTCF	coregulator		
	synergy in repression	recruitment of mSin3A-HDAC protein complex	Lutz et al, 2000
	synergy in activation	T3 iduced relief of enhancer blocking by CTCF	Lutz et al, 2003
	synergy in repression on negative TRE	unkown	Awad et al, 1999
P53	corepressor	interfer DNA binding ability of TR	Yap et al, 1996
19S regulatory particles	co-activator	promoting efficient and proper loading of liganded TR	Satoh T et al, 2009
Balal transcriptional factors			
TFIIB and TBP	corepressor	inhibiting PIC(preinixiation complex) formation	Fondell JD et al, 1996
TFIIF	co-activator	unkown	Liu Y et al, 2005
BTG1	co-activator	unknown	Busson et al, 2005

Table 2 Summary of TR Interacting Proteins Discussed.

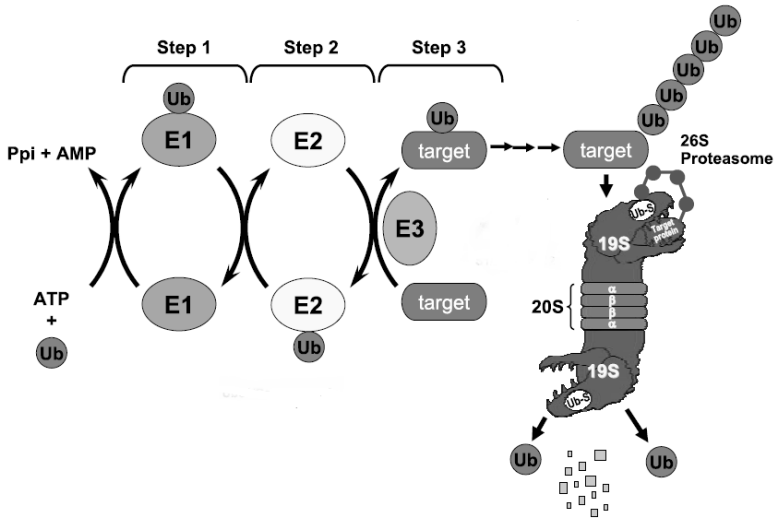
For the TR interacting proteins discussed above (Table.2), most of the interactions seem unnecessary at the first sight, further functional dissection of these interactions provided a more complicated and stringent auxiliary system for regulating TR activity. Although they are not present in the canonical picture of TR coregulatory proteins, some of these interactants (such as PP32 and TDG) turn out to regulate TR activity in a similar way by modifying the chromatin microenvironment surrounding the target genes, which is the general mechanism undertaken by the conventional regulators. There are also other TR interacting proteins that regulate TR activity using totally different ways which don't fit in the proposed canonical picture of coregulatory codes, exemplified by CTCF and 19S regulatory particles. Identification of these interacting proteins suggest that the accepted model is oversimplified and that the transcriptional regulation of TRs cannot be considered as an independent, chromatin-based process, but rather should be considered as coupled to many other cellular events that are carried out by distinct cellular proteins. There are other TR interacted proteins that were shown to modulate TR activity through unknown mechanisms, exemplified by BTG1, furthermore, new TR interactors are continually discovered (Hahm JB et al, 2014), further functional dissections of these different interactions should enable us to better understand how these different cellular proteins fine-tune each other to regulate TR actions.

2.5.2 Rapid Protein Turn-over of TRs

Accumulating studies indicate that the half-lives of many nuclear receptors, including thyroid hormone receptors, are controlled by ubiquitin-dependent proteolysis. Agonist binding attenuates receptor-mediated transcription by targeting the receptors for degradation. Ligand-induced receptor degradation has been reported for thyroid receptors (Dace et al. 2000), retinoic acid receptors (Kopf et al, 2000), retinoid X receptor (RXR) (Osburn DL et al, 2001), PPARα (Blanquart et al, 2002), PPARγ (Hauser et al, 2000), progesterone receptors (Lange et al, 2000), vitamin D receptor (Masuyama et al, 1998), and androgen receptor (Sheflin et al, 2000). This mechanism of receptor downregulation is largely conserved among the nuclear receptor superfamily and may be an important mechanism by which receptor signaling can be regulated.

Ubiquitination-Proteasome Pathway. The balance between protein synthesis and degradation is a critical and highly regulated process in a cell. One of the best characterized protein degradation pathway is ubiquitination-proteasome pathway. Proteins that are destined for degradation by the ubiquitin–proteasome pathway are covalently linked to a 76aa protein called Ubiquitin (Ub) that is highly conserved throughout eukaryotes. The process of Ub conjugation to other proteins called ubiquitylation, which is a signal for the subsequent degradation by the multisubunit ATP-dependent protease termed proteasome. Degradation of cellular proteins via the ubiquitin–proteasome pathway is a highly complex and tightly regulated process (Figure.12). Briefly, this pathway initiates via an ATP-dependent activation of free ubiquitin (Ub) by an ubiquitin-activating enzyme (E1). The activated ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2), which in turn transfer the ubiquitin directly to the target protein or to the third set of enzymes called ubiquitin-ligases (E3), E3 then targets the ubiquitin to a lysine residue of a specific protein substrate. The process is repeated to mark the protein substrate with a polyubiquitin chain, a recognition signal for the subsequent degradation by 26S proteasome complex.

The human genome reveals over 40 different potential E2s and over 500 different possible E3s (Wong et



al. 2003). It is the E3 alone or in combination with the E2 that determine the substrate specificity.

Figure 12 An overview of the ubiquitin–proteasome system (Kinyamu HK et al, 2005).
The ubiquitin pathway initiates with the activation of free ubiquitin (Ub) by ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to ubiquitin-conjugating enzyme (E2) and finally to a ubiquitin ligase (E3) which targets the ubiquitin to a lysine residue of protein substrate. The process is repeated to mark the protein substrate with a polyubiquitin chain, a recognition signal for the 19S and subsequent degradation of the protein by the 20S proteasome.

The proteasome holoenzyme, 26S proteasome, is a multi-subunit protein degrading machine and is composed of two sub complexes: a 20S proteolytic core particle and a 19S regulatory particle. The 20S core particle is sandwiched between two 19S regulatory particles. The 19S particle has two structural subunits: the 'lid' and the 'base', both are required for ubiquitinated substrate recognization. The 19S particle serves multiple roles for regulating the activity of the proteasome, including selecting substrates, removing the ubiquitin groups, unfolding the substrate and translocating the unstructured substrate to the 20S catalytic unit where it is degraded (Ciechanover, A et al, 1994, Hershko, A et al,1986).

The 20S catalytic core is a stack of four rings, two α and two β rings, the α-subunits play a role in the interaction of the 20S and 19S regulatory particle, while the β-subunits are functional proteolytic sites (Kinyamu, H. K et al, 2005). The special structure feature blocks the random degradation of proteins providing a high level of substrate specificity. Synthetic proteasome inhibitor MG132 has been used extensively to inhibit the proteasome activity while studying the effects of the ubiquitin–proteasome pathway on NR function.

Ubiquitination and TR activity. It was shown that T3 binding induces rapid proteasome-mediated degradation of thyroid hormone receptors. Specifically, T3 treatment did not enhance the ubiquitination, but rather triggered the degradation of ubiquitinated TR. However, the molecular basis underlying this triggering event is not clear. It has been suggested that this event could be mediated by the T3-induced

conformational changes of TR. as I mentioned before, the ubiquitinated substrates are unfolded and translocated through the 19S complex to be then degraded by the 20 S proteasome particle. T3 binding is known to induce dramatic structural reorganization of TR, which might facilitate the docking of the ubiquitinated TR onto the proteasome and facilitate the subsequential unfolding, translocation and proteasome degradation. Studies also showed that inhibition of proteasomal degradation causes increased total and ubiquitinated TR protein level, yet T3-dependent transcriptional activation were diminished, suggesting that the T3 induced TR degradation is essential for optimal T3-dependent transcriptional activity(Dace A et al, 2000, Kenessey A et al, 2005).

It was previously observed that many proteins contain PEST motifs, a sequence rich in proline, glutamic acid, serine, and threonine residues, which are involved in the recognition and degradation of target proteins by the ubiquitin-proteasome pathway. Phosphorylation of threonine and serine residues could contribute to the rapid degradation of PEST motifs-containing proteins (Rechsteiner M, 1996). Putative PEST motifs were identified within A/B and LBD domain of TRβ1 by sequence analysis (Dace A et al, 2000, Brunelle M et al, 2011), however, the putative PEST motifs seems not be involved in TRβ1 degradation (Brunelle M et al, 2011). The exact ubiquitination sites of TR require future studies.

2.5.3 TR Localization and Shuttling/TR Subcellular Trafficking

Most members of the nuclear receptor superfamily, especially for class I nuclear receptors, undergo subcellular trafficking upon ligand binding. For example, the unliganded glucocorticoid receptor are anchored in the cytoplasm through their association with heat shock proteins, while the liganded receptor undergoes rapid nuclear translocation and activates gene expression (Liu J et al, 2000), and, although unliganded PR (progesterone receptor), ER (estrogen receptor) and RAR (retinoic acid receptor) are primarily intranuclear, ligand binding is required for these receptors to interact with target genes (DeFranco DB et al, 1997).

As for TR, they localize primarily to the nucleus at steady state, where they repress gene expression in the absence of T3 and activate transcription in the presence of T3. Nuclear localization is critical for the gene regulatory function of TR. For example, cytoplasmic localization of TRα1 sequestered by its oncogenic homolog v-ErbA might contribute to its implications in oncogenesis (Bonamy GM et al, 2005). Though TRs were shown to have a dominant nuclear localization, cytoplasmic localization of TRs was also identified (Zhu XG et al 1998, Baumann CT et al, 2001). The cytosol TRβ1 was shown to complex with T3 and to interact with the p85 regulatory subunit of PI3K, leading downstream gene transcription (Cao X et al, 2005, Moeller LC et al, 2005), indicating a functional role of cytoplasmic localized TRs.

Accumulating evidence suggest that TR shuttles rapidly between the nucleus and cytoplasm, and this nucleocytoplasmic shuttling is crucial to the transcriptional regulation activities of TR. An important aspect of nucleocytoplasmic shuttling is mechanisms for transport of TR across the nuclear envelope. The nuclear envelope creates an intracellular compartment that enables spatial regulation of gene expression. Nuclear proteins cross the nuclear envelope via a large protein called nuclear pore complexes (NPCs) (Adams and Wente, 2013). The nuclear entry of TR is mediated by two co-existing pathway: TR can enter the nucleus by passive diffusion through the central channel of the NPCs, an

alternative, signal-mediated, energy-dependent import pathway also exists for TR (Caroline F et al, 2001). Signal-mediated transport requires soluble factors collectively called importins, to facilitate TR translocation into the nucleus. Importins bind to TR by recognizing a short lysine or arginine-rich amino acid motif known as a nuclear localization signal (NLS). Two NLSs in TR have been fully characterized: one in the N-terminal A/B domain (which is present in TRα1, but absent in TRβ1) and the other in the hinge domain (Manohara S et al, 2012).

Nuclear retention of shuttling proteins may depend upon the availability of intranuclear binding sites. It was shown that T3 treatment enhanced TR nuclear retention, suggesting that more intranuclear binding sites are available for the ligand-bound receptor (Caroline F et al, 2001). However, by checking the subcellular distribution of TRβ1 bearing mutations which destroy its interaction with NCoR/RXR or its binding ability to DNA, Christopher T et al showed that interactions of TRβ with various cofactors, rather than specific DNA interactions, play the predominant role in determining the intracellular distribution of the receptor (Christopher T et al,2001).

Once inside the nucleus, nuclear receptors are faced with the task of locating onto the chromatin, the appropriate subnuclear trafficking of receptors is an integral component of transcriptional control. The classic view is that the receptor binds to a recognition site and remains at that site for as long as the ligand is present in the cellular milieu (Becker P et al, 1984). However, many studies provide direct evidence that the hormone-occupied receptor undergoes rapid exchange between chromatin and the nucleoplasmic compartment (Rigaud G et al, 1991, James G et al, 2000). The receptor may interact transiently with a response element, recruiting a secondary set of factors that in turn form a stable complex at the regulatory site. This type of mechanism has been referred to as “hit and run” and has been proposed for many members of nuclear receptors.

TR exits the nucleus through two pathways, one dependent on the export factor CRM1 (chromosome region maintenance 1) and the other CRM1-independent (Grespin et al, 2008). Three nuclear export signal (NES) motifs were characterised in TR, located separately in helix 3, helix 6 and helix 12 within LBD domain of TR (Manohara S et al, 2012).

Eukaryotic cells form discrete compartments, which allow gene expression to be regulated by altering the subcellular distribution of transcription factors in response to external stimuli. To fully understand the cellular response to T3, investigation of all levels of receptor control is essential, including regulation for transport of TR across the nuclear envelope and its subsequent nuclear retention, chromatin recruitment and dissociation and the final nuclear export.

2.5.4 Post-translational Modifications (PTMs) of TR

A wealth of data have enlightened the role of posttranslational modifications (PTMs) in modulating the functions of NRs, the transcriptional activity of TR is modulated by post-translational modifications such as phosphorylation, sumoylation and acetylation.

Phosphorylation

Long before the uncovering of the underline mechanisms and the identification of phosphorylation sites within TRs, several groups have observed that TR phosphorylation can enhance T3-mediated transcriptional activation of target genes (Lin KH et al, 1992; Jones KE et al, 1994). Consistently, it has been demonstrated that TR can be phosphorylated both *in vitro* and *in vivo* (Lin KH et al, 1992; Sugawara et al, 1994). Furthermore, TR phosphorylation has been shown to modulate TR action by modulating TR complex binding to DNA, influencing TR steady state and regulating TR cofactors interactions.

Studies have been made to explore the influence of TR phosphorylation on TR binding affinity towards DNA. Sugawara et al. observed that phosphorylation selectively enhanced TR homodimer, but not TR/RXR heterodimer, binding to several different TREs (Sugawara et al, 1994). Bhat et al. showed that phosphorylation enhanced DNA binding of both TR homodimer and TR/RXR heterodimer (Bhat et al, 1994). Interestingly, Tzagarakis-Foster C et al. observed that TR phosphorylation by PKA (protein kinase A) significantly inhibit the ability of TR to bind DNA as monomers (Tzagarakis-Foster C et al, 1998). Collectively, these results suggest that phosphorylation, in addition to T3 binding, could modulate TR complex binding to TREs. Another interesting study from Katz D et al. showed that functional regulation of TR variant TRα2 by phosphorylation relates to influencing its DNA binding ability. TRα2 was shown to inhibit TR-dependent transactivation involving competition for T3 response elements. Katz D et al. showed that TRα2 could be phosphorylated by casein kinase II (CKII) on serines 474 and 475. This serine phosphorylation occurs in mammalian cells and reduces the inhibitory activity of TRa2 towards wild type TR by inhibiting its binding to DNA. Mutation of the two target serines to alanine restores the DNA binding of TRa2 and its dominant negative activities. Consistently, a more recent study showed that phosphorylated TRα2 is primarily cytoplasmic, whereas unphosphorylated TRα2 is primarily nuclear, suggesting a role of phosphorylation in withholding TRα2 in the cytoplasm (Xu B et al, 2005). These results imply that phosphorylation may provide a rapid, T3-independent mechanism for cell-specific modulation of the expression of T3-responsive genes (Katz D et al, 1995).

It has been shown that phosphorylation of TR also plays a critical role in its activity and protein stability. Inhibition of protein phosphatase 1 and 2A with okadeic acid (OA) not only significantly potentiates the transcriptional activation ability of TRs, but also increases their protein stability (Ting YT et al, 1997; Lin KH et al, 1992), although this stabilization effect induced by OA seems to be cell-type specific and also TR isotype specific (specific for TRβ1, but not TRα1 or TRβ2). Subsequently, Chen SL et al. demonstrated that MAPKs are possible candidates responsible for the nuclear phosphorylation of TR and are critical factors modulating the transcriptional activity and protein stability of TR subsequent to ligand binding. Manipulating MAPKs activity by overexpression or inhibitors could modulate TR activity and protein stability (Chen SL et al, 2003).

Moreover, it has been reported that serine phosphorylation of TRα1 by T4-activated MAPK results in dissociation in the cell nucleus of TR and silencing mediator of retinoid and thyroid hormone receptor (SMRT), leading to the de-repression of TR transcriptional activity (Davis PJ et al, 2000). A further study identified the serine 142 within the DBD site of TRβ is the likely target site of T4-activated MAPK phosphorylation and that the docking site on TRβ for activated MAPK includes residues 128-133

(KGFFRR), a basic amino acid-enriched motif novel for MAPK substrates. Accordingly, TR mutations in the proposed MAPK docking domain and at residue 142 modulated T4 induced release of co-repressor and recruitment of co-activator proteins by the receptor to a different extent, possibly due to the different impairment of TR structure introduced by different mutants (Lin HY et al, 2003).

These findings suggest that phosphorylation potentially may regulate diverse and important TR functions, although the detailed mechanisms underline these modulations remain to be elucidated.

Sumoylation

Sumoylation requires conjugation of SUMO (small ubiquitin-like modifier) to lysine residues in specific target proteins via an enzymatic cascade: SUMO proteins are first activated in an ATP-dependent manner by the activating enzyme E1, SUMO is then passed to the active site cysteine of the conjugating enzyme Ubc9 (ubiquitin-conjugating 9), SUMO is finally covalent attached to the target protein with the help of E3 ligases, which have substrate specificity (Wilkinson KA et al, 2010). Sumoylation can influence the function of a transcription factor by modifying protein conformation, the interface of protein-cofactor interaction, DNA binding, and ligand binding (Geiss-Friedlander R et al, 2007).
Studies showed that TR sumoylation is essential for TR-mediated gene regulation. TRα and TRβ are modified by specific SUMOs at different sites with distinct E3 ligase preferences and differ in their response to the ligand. TRα1 is sumoylated at lysines 283 and 389 and TRβ at lysines 50, 146, and 443. TRα prefers E3 ligase PIASxβ, and TRβ prefers E3 ligase PIAS1. TRβ, but not TRα, requires T3 for sumoylation (Liu YY et al, 2012; Weitzel JM et al, 2016), however, a preferential sumoylation of TRβ in the presence of T3 was not observed in the latter study. These findings demonstrate that conjugation of SUMO to TR has a TR-isoform preference, K389 (TRα) and K443 (TRβ) turned out to be orthologous residues, lysine residues K283 (TRα) and K50 (TRβ) are isoform-specific sumoylation sites (Weitzel JM et al, 2016). Therefore, specifically targeting K283 in TRα or selectively aiming K50 in TRβ might offer a way to differentially regulate the two TR isoforms. Furthermore, SUMO conjugation to TR modulates T3-mediated gene regulation on both positive and negative TREs by modifying TR interactions with cofactors. TR sumoylation was shown required for the T3-induced recruitment of the co-activator CREB-binding protein (CBP) and release of nuclear receptor co-repressor (NCoR) on a positive TRE, as well as T3-mediated recruitment of NCoR and release of CBP from the TSHβ-negative TRE (Liu YY et al, 2012). Consistently, TR sumoylation mutants have been shown to impair preadipocyte differentiation and proliferation though down-regulation of C/EBPs, constitutive interaction with NCoR, interference with PPARγ signaling, and disruption of the Wnt canonical signaling pathway, indicating an essential physiological role of TR sumoylation (Liu YY et al, 2015). All these results indicate that sumoylation contributes to the fine-tuning of TR effects on cellular and physiological homeostasis.

Acetylation

Acetylation was also shown to be an important post-translational modification in regulating NRs actions. Since the initial identification of NR acetylation with the estrogen receptor alpha (ERα) and androgen receptor (AR) a decade ago (Fu M et al, 2000; Wang C et al, 2001), emerging evidence has demonstrated that other NRs are acetylated as well, including thyroid hormone receptor, liver X receptor, retinoid X

receptor and glucocorticoid receptor. Acetylation-mediated regulation of NRs includes transactivation, subcellular localization, DNA binding, stability and degradation, ligand binding, and cofactor binding (Sanchez-Pacheco A et al, 2009; Lin HY et al, 2005; Li X et al, 2007; Fang S et al, 2008; Kemper JK et al, 2009; Zhao WX et al, 2007; Kim MY et al, 2006; Ito K et la, 2006; Gaughan L et al, 2002).

Lin HY et al. has demonstrated that T3 or T4 treatment leads to accumulation of acetylated TR, this acetylation process requires the activation of ERK1/2 MAP kinase. The acetylation sites are mapped to the protein translocation domain (D-domain) between amino acid 128 and 142, which are known to be relevant to the MAPK docking site (Lin HY et al, 2005). Detailed mapping identified three conserved lysines 128, 132, and 134 as acetylation target sites of the cAMP-response element-binding protein acetyltransferase (Sanchez-Pacheco A et al, 2009). The ligand binding induced TR acetylation increases TR/RXR heterodimeric binding to its cognate element, while the acetylation mutant of TR is defective in both coactivator recruitment and corepressor dissociation. Although the wild type TR binds to T3 ligand efficiently, the acetylation mutant exhibits no detectable binding activity, suggesting a role of TR acetylation in regulating TR DNA binding, ligand binding and cofactor binding. Additionally, the acetylation mutant of TR has lost the ability to show increased inhibition of fibroblast transformation by oncogenic Ras in the presence of hormone, implying an important role of TR acetylation in its antioncogenic actions (Sanchez-Pacheco A et al, 2009).

The HDACs mediated deacetylation of acetylated NRs was also shown to regulate NR activities (Li X et al, 2007; Fang S et al, 2008; Kemper JK et al, 2009; Kim MY et al, 2006; Ito K et la, 2006; Gaughan L et al, 2002). Recently, an interesting study revealed a direct interaction between TR and SIRT1, which is a nicotinamide (NAD+)-dependent deacetylases. Further dissection of this interaction uncovered a coactivator function of SIRT1 in regulating TR activity by promoting TR deacetylation and enhancing ubiquitination-dependent TR turn-over. However, unlike general TR coactivators such as the SRCs, which are needed for T3 activation of most TR target genes (Fondell JD et al, 2013), SIRT1 enhances TR activity in a strongly gene-specific manner, SIRT1 knocking down only strongly inhibit T3 response of a subset of TR target genes (Suh JH et al, 2013). A similar study also indicate that SIRT1 is required for optimal T3 response of endogenous TR-regulated genes in cultured liver cells, in this study, they proposed that SIRT1 could promote T3 dependent gene activation indirectly by promoting deacetylation of PGC1α (a coactivator for TR) and enhancing its activity (Thakran S et al, 2013).

Collectively, TR acetylation is an important post-translational modification in regulating TR activity, the acetylation state of TR could be reversely regulated by histone acetylase CBP/P300 and the deacetylase SIRT1. We therefore suggest that TR acetylation contribute to TR mediated transactivation upon T3 treatment and SIRT1 subsequently deacetylates TR to trigger its ubiquitination and turnover at the end of TR activation, thus the separate acetylation and deacetylation steps may be important components of the transcription cycle. To fully investigate this idea, it will be important to understand the kinetics of recruitment of different HATs/HDACs, the role of different TR acetylation sites in T3 response and the correlation of these events with TR acetylation status and transcriptional activity.

2.6 Non Genomic Actions of Thyroid Hormone

TH has non-genomic effects that occur within seconds to minutes, this mode of action relies on the generation of second messengers such as Ca2+ and cAMP stimulated by T3, which influences the Akt and PKC signaling pathways, leading to a modification of gene expression (Cheng SY et al, 2010).

The non-genomic actions of TH can be exerted in a T3 independent manner. It is proposed that thyroid hormone (T3 or T4) binds to plasma membrane receptor integrin αvβ3, the thyroid hormone signal is then transduced to MAPK (ERK1/2) through phospholipase C (PLC) and protein kinase C (PKC) (Davis PJ et al, 2008). Hormone-activated ERK1/2 promotes phosphorylation and nuclear translocation of specific proteins resident in cytoplasm, including estrogen receptor (ERα) (Lin HY et al, 2007), TRβ1 (Davis PJ et al, 2000, Cao HJ et al, 2009), STAT1α (signal transducing and activator of transcription 1α) (Lin HY et al, 1998), leading downstream regulation of specific gene transcription. Cellular events induced from the cell surface αvβ3 receptor include angiogenesis (endothelial and vascular smooth muscle cells) (Bergh JJ et al, 2005) and tumor cell proliferation (Davis FB et al, 2006) and tumor xenografts (Yalcin M et al, 2008). A recent study showing that T3 could act as an inhibitor of a ketimine reductase, Crym, which has been initially considered as a component of a storage compartment for T3 (Mori et al, 2002; Suzuki et al, 2007; Hallen et al, 2015), provided another possibility of TH action that T3 simply acts as an allosteric regulator of a metabolic enzyme.

The second type of non-genomic response relies on alternate functions of TRs. Recently, a 30KDa plasma-membrane localized receptor P30 were identified as a high affinity TH receptor responsible for stimulating Akt pathway from the plasma membrane (Kalyanaraman H et al, 2014).This signaling mechanism has been shown occurs in multiple cell types, and is physiologically relevant, enhancing osteoblast proliferation and survival in vitro and bone formation in vivo. A previous study proposed that TRα1 was also able to trigger the same type of cellular response by indirectly interacting with P85α subunit of PI3K (Hirot et al, 2006). However, another study concluded that neither TRα1 nor P30, but TRβ1 is responding to T3 at plasma membrane. Unlike TRα1, which lacks a critical tyrosine residue, TRβ1 can serve as an intermediate between tyrosine kinase receptors and the PI3K/Akt pathway (Storey et al, 2006; Martin et al, 2014). Several possibilities mentioned above received experimental supports, which all ultimately converged on the rapid activation of the PI3K/Akt pathway by T3. Moreover, a 43kDa TR isoform has been identified as a mitochondria protein (Wrutniak C et al, 1995), it still possesses the ability to bind DNA and has been proposed to act as a regulator of mitochondrial genome expression (Blanchet E et al, 2012).

As discussed above, several proposed pathways for non-genomic signaling are compatible with an intervention of thyroid hormone receptors, including the full-length and the truncated TR isoforms. Furthermore, GilIbañez P et al. observed that neurons without TR do not display any residual transcriptional response to T3 (GilIbanez P et al, 2015), implying an important role of TR in non-genomic effect of TH. The involvement of TR also suggests that the genomic and non-genomic responses are not entirely independent pathways.

2.7 TR Mutations and RTH

2.7.1 Clinical Features of Resistance to Thyroid Hormones

The critical role of TRs is evident in that mutations of the TR genes cause resistance to thyroid hormone syndrome (RTH). RTH is a syndrome characterized by decreasing sensitivity of the pituitary and/or other target tissues to the action of thyroid hormones. According to the mutated TR genes, RTH is classified as RTHα and RTHβ (Refetoff S et al, 2014).

Resistance to Thyroid Hormone beta (RTHβ), a dominantly-inherited disorder due to *THRB* mutations, is characterized with persistent elevated serum free thyroid hormone levels (thyroxine (T4) or triiodothyronine (T3)) and nonsuppressed TSH. Other clinical signs are goiter, affected vision and hearing, tachycardia, attention deficit/hyperactivity disorder, decreased IQ, and dyslexia (Weiss RE et al, 2000, YEN PM et al, 2003) (Figure.13).

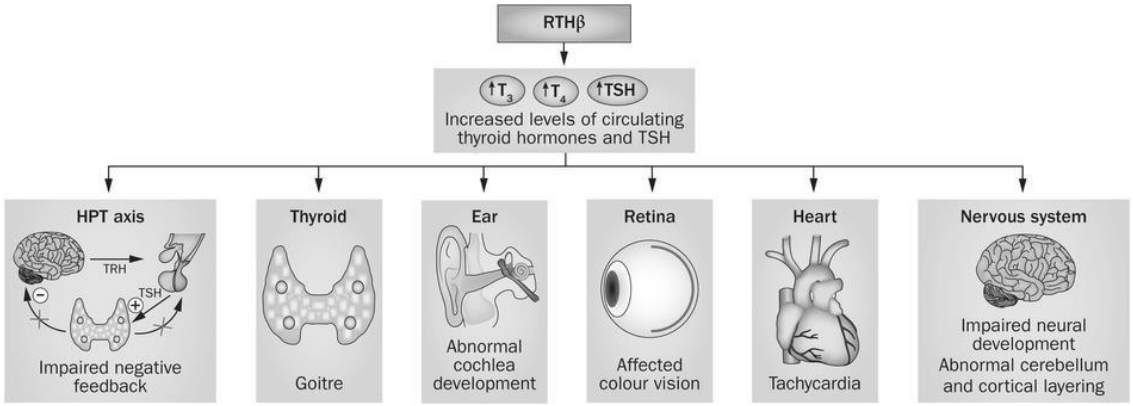


Figure 13 Overview of tissues and homeostatic functions affected in RTHβ (Tânia M et al, 2014).
RTHβ is characterized with increased levels of circulating thyroid hormones and TSH, which results from the impaired HPT (hypothalamic–pituitary–thyroid) axis function, other symptoms, including goiter, abnormal cochlea development, affected colour vision, tachycardia and nervous system defects, are also a reflection of TRβ expression in the target tissues (Thyroid, ear, retina, heart and nervous system).

In the past 5 years, reports have emerged of patients with mutations in *THRA* that lead to the development of Resistance to Thyroid Hormone alpha (RTHα) (Bochukova E, et al,2012, van Mullem A et al,2012, Moran et al,2013,2014). Affected individuals are heterozygous for *THRA* mutations. RTHα is biochemically characterized by raised/high-normal T3 and low/low-normal T4 levels, resulting in a markedly reduced T4/T3 ratio together with low rT3 levels in some cases; Clinical features include dysmorphic facies, skeletal dysplasia (macrocephaly, epiphyseal dysgenesis), growth retardation, constipation, bradycardia, dyspraxia and intellectual deficit (Nadia Schoenmakers et al, 2013) (Figure.14). Overall, RTHα and RTHβ patients display a different set of symptoms that mostly reflect the different patterns of expression for TRα and TRβ (Figure.11 and 12). RTHβ patients present a hypothyroid phenotype (e.g. impaired HPT axis) in tissues (e.g. the pituitary) that express the mutant TRβ, whereas the elevated serum hormone causes hyperthyroidism (e.g. tachycardia) in tissues that mainly contain TRα1 (e.g. heart). RTHα patients with TRα1 mutations exhibit hypothyroid features (e.g. skeletal dysplasia, reduced intestinal motility, low heart rate and basal metabolic rate) in tissues (bone,

gastrointestinal tract, myocardium, skeletalmuscle) expressing predominantly TRα, whereas, organs containing mainly TRβ remain hormone sensitive(e.g. hypothalamus, pituitary and liver).

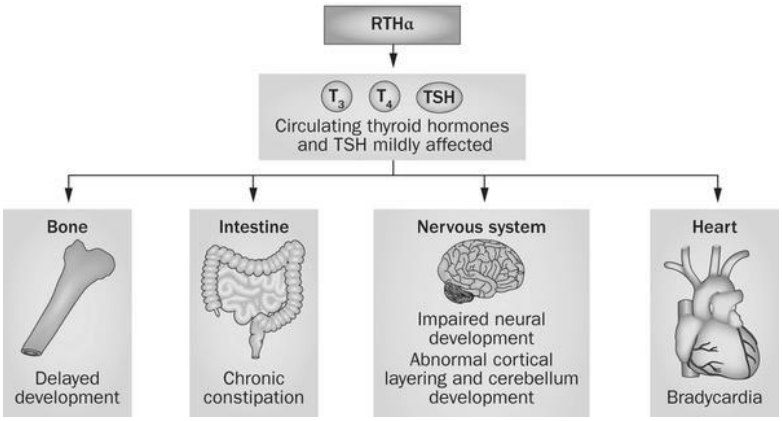


Figure 14 Overview of tissues and homeostatic functions affected in RTHα (Tânia M et al, 2014).
In RTHα patient the levels of circulating thyroid hormones are mildly affected. Additionally, these patients have delayed bone development, heart defects, chronic constipation and impaired neuronal development.

The clinical symptoms of RTH (both RTHα and RTHβ) is highly variable relates to the range of symptoms and metabolic consequences of TR mutations. Most individuals are clinically euthyroid, some individuals may appear to be hypothyroid while others may appear hyperthyroid. Furthermore, the same subject with RTH may exhibit both symptoms of hypothyroidism and hyperthyroid. The clinical phenotype of RTH also vary between families with different TR mutations, between families with the same mutation, however, individuals between members of the same family with identical mutations also display different phenotype (Roy E. Weiss et al, 2000). One explanation is that the milieu of cofactors for TR within a specific microenvironment for TR target genes regulates the ultimate expression. That is to say that the cofactors modulate the thyroid hormone response based on the nature and the level of the corepressors or coactivators expressed in a particular tissue.

2.7.2 Molecular Basis of RTH

The concept of hormone resistance was introduced by Fuller Albright et al with the description of pseudohypoparathyroidism (Albright F et al, 1937). In the case of RTH, it was difficult to find the precise cause of the hormonal resistance, until the cloning of the thyroid hormone receptor TRβ gene by Weinberger et al in 1986. A link between the phenotype of RTH and the TRβ gene mutations were established thereafter (Usala SJ et al, 1988, Sakurai A et al, 1989, Takeda K et al,1992). The recent discoveries of genetic defects that reduce the TH (thyroid hormne) effectiveness through altered cell-membrane transport (Dumitrescu AM et al, 2004, Friesema EC et al, 2004) and metabolism (Dumitrescu AM et al, 2005) have broadened the definition of RTH from TH insensitivity to all defects that can interfere with the biological activity of TH.

RTH is mostly caused by mutations in TR genes. The incidence of RTHβ is around 1 in 40,000, and with around 170 different receptor mutations known to be associated with RTHβ. Patients with single amino acid substitution/deletion, frameshift mutations, and truncations of TRβ have been identified. Mutations are located in the carboxyl terminus of the receptor, mostly contained in three 'hot spots' located within the LBD and the adjacent hinge region, and are distributed into three clusters (Figure.15) (Weiss RE et al. 1993, Adams M et al, 1994, Collingwood TN et al, 1998). Though limited number of TRα mutations associated with RTHα has been identified until now (14 cases from 10 different families), frameshift/premature stop mutations or missense, amino acid changes have been identified for TRα mutations among RTHα patients. Most cases harbour mutations, which selectively disrupt the carboxyterminal activation domain of TRa1 (Bochukova E et al, 2012, van Mullem A et al,2012, Moran C et al, 2014, Tylki-Szymanska A et al, 2015). Almost all TR mutants showed reduced affinity for thyroid hormone.

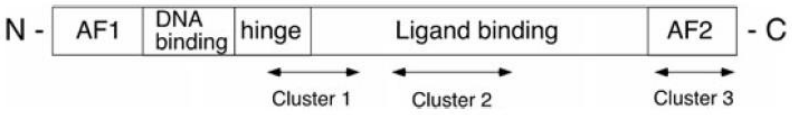


Figure 15 Schematic view of RTH mutation clusters within thyroid hormone receptor

According to the identified mutations in TR genes associated with RTH, most mutations are located in three 'hot spots': one spans the hinge region and the beginning of LBD domain, another two are located within the LBD domain. Though limited number of TRα mutants (associated with RTH) is identified, most of the mutations are located at the C terminus of the LBD domain.

Most patients are heterozygous, with both wild type (WT) and mutated allele of TR gene. Analyses of the TRβ gene mutations among RTH patients showed that individuals expressing a single WT TRβ allele due to deletion of one allele are normal, whereas those expressing both a WT and a mutant allele have RTH, suggesting that the presence of a defective TRβ interferes with the normal function of the WT counterpart, a phenomenon termed dominant negative effect (Yen PM et al, 1994). The mechanisms for this dominant negative effect likely involve follow aspects: (i) the formation of inactive dimers, including homodimers of the mutant receptors and heterodimers with RXR (PIEDRAFITA FJ et al, 1995); (ii) the competition for DNA binding of the transcriptional inactive dimers (NAGAYA T et al, 1992); the binding to TREs by inactive mutant homodimers or TR/RXR heterodimers that cannot bind T3 and hence cannot activate transcription of target genes, instead, some TR mutants constitutively repress basal transcription even in the presence of T3 (PIEDRAFITA FJ et al, 1995). (iii) the impairment of T3 induced corepressor release or interaction with coactivators (SAFER JD et al, 1998, Collingwood TN et al, 1998).

The amount of dominant negative activity and severity of clinical phenotype of TR mutants correlates with impairment of in vitro T3 binding and the impairment of corepressor dissociation in the presence of T3 (REFETOFF S et al, 1993). Studies on TRβ R320H mutant have better elucidated this issue, the R320H mutant showed threefold lower T3 binding affinity than wild-type TR, however, it was able to release NCoR and recruit SRC-1 at higher T3 concentrations. Consequently, this mutant had potent dominant negative activity at low T3 concentrations but weaker dominant negative activity at higher

concentrations (Liu et al, 1998). The amount of dominant negative activity by a mutant TR also depends in part on the level of mutant receptor expression. For example, a patient who was homozygous for mutations in both TRβ alleles had severe RTH and mental retardation. In contrast, his parents who had mutations in only one TRβ allele had mild RTH (ONO S et al, 1991).

2.7.3 Modelling RTHα

In recent years, studies have emerged of patients with mutations in *THRA* that lead to the development of RTHα. The first patient identified with RTHα carried a heterozygous nonsense mutation in *THRA* (E403X) that resulted in the introduction of a premature stop codon leading to a truncated helix 12. Structural modelling suggested that the E403X mutation removes an α-helix at the carboxyterminus of TRα1, thereby enhancing corepressor recruitment but abrogating coactivator binding. Functional studies of E403X mutant confirmed these properties. Consistent with this, E403X mutant did not bind T3 and exhibited negligible hormone-dependent transactivation. A potent inhibition of transcriptional activity of wild type TRα1 was observed when they were coexpressed, such dominant negative effect of the mutant receptor *in vitro* correlated with impaired thyroid hormone action in patient-derived cells studied *ex vivo*, with markedly reduced T3 response of a known TR target gene (KLF9), suggesting that such dominant negative inhibition operates *in vivo* (Bochukova E et al, 2012). This patient presented with classic features of hypothyroidism (growth retardation, developmental retardation, skeletal dysplasia, and severe constipation) but paradoxically only borderline-abnormal thyroid hormone levels.

Prior to the identification of human cases, mouse TRα knockout and knockin models were invaluable in predicting the likely phenotype of RTHα patients and its pathogenesis. Several mutant *THRA* mouse models have been developed, containing mutations found in the *THRB* locus in patients with RTHβ or similar to such, and findings from experiments using these mice are conflicting. However, four models that produced similar phenotypes to those seen in patients with RTHα, including TRα1-PV, TRα1-L400R, TRα1-P398H and TRα1-R384C.

The TRα1-PV mutant knock-in mouse model was developed to mimic a human *THRB* mutation that has a frameshift of the C-terminal 14aa of TRβ1 and results in severe resistance to thyroid hormone. The TRα1-PV mutant lost its binding ability to T3 and showed strong dominant-negative activity. The TRα1-PV mutant mice were dwarfs and exhibited mild thyroid failure (Kaneshige M et al, 2001).

The TRα1-L400R knock-in mouse was developed on the basis of an artificial RTH mutation (TRβ L454R). This mutation prevents the binding of co-activators but preserves interaction with co-repressors. In contrast to the TRα1-PV mutant, TRα1-L400R binds T3 normally but retains strong dominant-negative activity. In addition to dwarfism, these mice have delayed cerebellar development, which is characterized by a delayed granule-cell differentiation pattern similar to that seen in patients with congenital hypothyroidism. These mice have difficulty maintaining body temperature under stress and usually do not survive beyond 3 weeks after birth (Quignodon L et al, 2007).

The TRα1-R384C knock-in mouse model was developed to mimic a human *THRB* mutation (TRβ R438C) that resulted in decreased T3 affinity by 10-fold. These mice exhibited slightly lowered serum levels of T3 and T4. Heterozygous mice exhibit severe retardation of post-natal development and growth, while

adult mice overcome most of these defects except for cardiac function abnormalities (Tinnikov A et al, 2002).

The TRα1-P398H mutant, with a proline to histidine substitution, is developed based on the TRβ mutant P449H associated with resistance to thyroid hormone (RTHβ). Similar to TRα1-R384C mutant, the TRα1-P398H was shown to interfere T3 binding and function as a dominant-negative receptor. TRα1-P398H mutant mice have slightly elevated serum triiodothyronine (T3) and thyroxine (T4), and elevated serum thyrotropin (TSH) concentrations, they also showed decreased heart and increased body fat accumulation and elevated serum levels of leptin, glucose, and insulin. In addition, there is a marked impairment in sympathetic mediated lipolysis in white adipose tissue (Liu YY et al, 2003).

Phenotype	TRα1-E403X	Mouse model
Low T4/T3 ratio	+	TRα1-PV
Low rT3	+	NA
Low IGF1 (insulin-like growth factor 1)	+	NA
Growth retardation	+	TRα1-PV;TRα1-L400R;TRα1-R384C
Delayed bone age, impaired ossification	+	TRα1-PV;TRα1-L400R;TRα1-R384C
Macrocephaly	+	TRα1-PV
Reduced GI motility	++	TRα1-L400R
Motor abnormality	+	TRα1-L400R;TRα1-R384C
Cognitive impairment	+	TRα1-R384C
cardiovascular defects, low heart rate	+	TRα1-P398H ; TRα1-R384C
	+ present; ++ severe	

Table 3 Comparison of human and murine mutant TRα1 phenotypes

Collectively, all four mutations lead to reduced or abolished T3 binding and recruitment of co-activators and caused a dominant negative effect. Interestingly, despite having similar biochemical properties *in vitro*, each of these mutations resulted in slightly different *in vivo* phenotypes. It is noticeable that these three mutations- TRα1-PV, TRα1-L400R and TRα1-P398H- are all located in helix 12, which in wild type TRα1 undergoes dramatic shift in position upon T3 binding, suggesting that the structural reorganization of helix 12 in TRα1 could be sensitive to mutational alteration. Previously, it has been shown that in vitro different TRβ mutants associated with RTHβ interact differently with corepressors (Yoh SM et al, 1997, Tagami T et al, 1998). Although, limiting knowledge about how these TRα mutants interact with various coregulators *in vivo* for the moment, it is conceivable that a different mode of interaction of TRα1 mutants with various coregulators could lead to differential transcriptional regulation of different target genes, resulting in the manifestation of different phenotypes.

Establishment and studies of the different TRα1 mutant mice models helped us to better predict the pathogenesis of patients suffering from RTHα. The markedly reduced T4/T3 ratio together with decreased rT3 levels in some cases found in RTHα patients may reflect altered metabolism of thyroid hormones in these patients. One possibility is that, as has been documented in TRα1-PV mice, increased hepatic type I deiodinase (D1) levels augment T4 to T3 conversion; alternatively, reduced tissue levels of type III deiodinase (D3), whose expression is TRa1 regulated (Barca-Mayo O et al, 2001), may contribute

to these abnormalities, as D3 was responsible for catalyzing inner-ring deiodination of T4 to rT3 and T3 to T2.

Moreover, many features (skeletal abnormalities, reduced colonic motility, bradycardia, low heart rate and basal metabolic rate) seen in mutant TRα1 (E403X) patients are mirrored (Table 3) in these four targeted mutant mice. The functional properties of murine TRα1-PV mutant is most similar to human E403X mutant; consistent with this, the clinical features in our E403X -mediated RTHα case most closely resemble the phenotype of TRα1-PV mice.

3 Retinoic Acid Receptor

Retinoic acid (RA), the major bioactive metabolite of vitamin A, plays an essential role in cell growth and differentiation that are relevant to embryonic development and adult physiology. RA activity is mediated primarily by members of the retinoic acid receptor (RAR). RAR belongs to the nuclear receptor (NR) superfamily of transcription factors. Three RAR genes (*RARA, RARB, RARG*) encode three isotypes RARα, RARβ and RARγ. The expression level of different isoforms varies among tissues. Similar to other nuclear receptors, RAR display a modular structure with six distinct regions (denoted A-F) with three function domains: the N-terminal A/B domain, and the evolutionary conserved DNA binding domain (DBD) and ligand binding domain (LBD) (Figure.16).

RARs form heterodimers with members of the retinoid X receptor (RXR) subfamily and act as ligand-regulated transcription factors through binding specific RA response elements (RAREs) located in target genes promoters. RAREs are typically composed of two direct repeats of the core hexameric motif PuG(G/T)TCA (di Masi A et al, 2015). The classical RARE is composed of the two direct repeats (DRs) spaced by 5bp (referred to as DR5). However, the heterodimers also bind to direct repeats separated by 1bp (DR1) or 2bp (DR2) (Balmer J E et al, 2005). Additionally, a few instances of non-canonical RAREs were also characterized. They exhibit degenerate core half-site and/or non-classical spacer like DR8 (Chang Y S et al, 2006), DR3 (Fujisawa K et al, 2000), ER8 (Carter M E et al, 1994) and IR0 (Lee C H et al, 1999). The DR0, DR8, and IR0 response elements were identified as the most abundant RAR binding elements in mouse embryoid bodies or murine embryonic carcinoma F9 cells. More recently, whole genome analyses have drawn a more precise RAR/RXR binding picture in various cell types including ES cells (Mendoza-Parra MA et al, 2011, Moutier,E et al, 2012, Gerard Benoit et al, 2015).

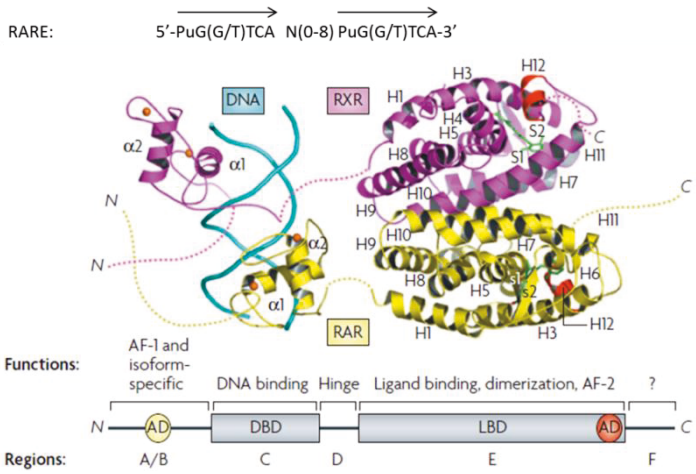


Figure 16 Structural and functional organization of retinoid receptors and schematic representation of RARE (de Lera AR et al, 2007).

RARE is composed of a direct repeat of the consensus motif 5'-(A/G)G(G/T)TCA-3', spaced by 0 to 8 nucleotides. Like other nuclear receptors, retinoic acid receptors (RARs) consist of six structural and functional domains denoted A to F. The DBD is composed of two zinc-finger motifs and two α helix, that mediates sequence-specific DNA recognition (The orange spheres in the DBD indicate Zn2+ ions). Transcriptional regulation is mainly mediated by the multifunctional LBD, which contains a ligand-binding pocket, a dimerization surface and a ligand-dependent transcriptional activation function (AF-2). LBD domain contains 12 α-helices, the C terminal most helical segment, named H12, is shown in red in each subunit.

The classical model of nuclear receptor action postulates that the major effect of ligand binding relates to convert the preexisting DNA-bound NR dimers from a repressive complex to an active complex, exemplified by TR (refer to TR part). However, studies of RAR activity in both embryonic stem (ES) cells and embryonal carcinoma F9 cells (which I will discuss later in cell models part) showed that the most evident consequence of RA stimulation is a massive increase in the number of RAR/RXR bound genomic loci (Mahony S et al, 2011; Gerard Benoit et al, 2015). Hence, the widely accepted mechanism of repression by unliganded RAR operates at most on a limited fraction of the retinoid target sites. Furthermore, the ligand induced transcriptional activation of RAR is closely correlated to ligand induced DNA binding of RAR. However, how RA promotes RAR/RXR recruitment to DNA is still an open question.
RARs are also involved in other nongenomic biological activities such as the activation of translation and of kinase cascades (refer to Rochette-Egly C et al, 2013 for more details). These kinases target RARs and their coregulators, adding more complexity to RAR-mediated transcription. The nongenomic effects of RA cooperate with the genomic effects for fine-tuning RAR activity.

3.1 RA Induces Stem Cell Differentiation via Epigenetic Changes

Retinoic acid (RA) is a well-known regulator of embryonic development as well as adult physiology. At the cellular level, RA treatment triggers biological process such as growth arrest, differentiation and apoptosis. Stem cell differentiation is a process that involves a series of epigenetic changes, RA exerts

potent effects on stem cell differentiation in part via the modulation of the epigenome. These epigenetic changes, including histone modifications and DNA methylation, could be stable and heritable changes.

RARs mediate most of the actions of RA in stem cells (Gudas LJ et al, 2011). Activation of transcription is the first step in the RA associated differentiation process. It occurs rapidly, within minutes to a few hours after RA addition. RA addition triggers the binding to DNA of RAR/RXR hetreodimers, and the activation of RA response genes, such as homeobox gene Hoxa1, Cyp26a1 and RARβ2 (Gerard Benoit et al, 2015). In some cell types, RA addition also triggers persistent mitogen activated protein kinase signaling to induce differentiation (Congleton J et al, 2012). Coregulatory proteins, including co-activators and co-repressors, recruited to the RXR/RAR complex are also involved in regulating the sensitivity of cells to RA's differentiation-inducing effects by regulating RAR activity (refer to NR coregulator part). Despite its importance, much less research has focused on how RA signaling leads to transcriptional repression. Studies showed that transcription repression by RA in ES cells is often mediated by an increase in the expression of the orphan receptor GCNF (germ cell nuclear receptor), which then repress pluripotency genes such as Sox2, Nanog and Oct4 (Gu et al, 2005; Akamatsu et al, 2009).

Polycomb group-repressive complexes (PRC1 and PRC2) are key proteins controlling the transition from stem cells to differentiated cells (Boyer LA et al, 2006 ; Pasini D et al, 2007), they also serve as important mediators of the epigenetic changes induced by RA. The PRCs confers transcriptional repression via the placement of the repressive H3K27me3 histone mark. Indeed, the protein Ezh2, a component of the PRC2 complex, places the H3K27me3 mark recognized by PRC1 factors, which in turn mono-ubiquitinylate histone H2A lysine 119 (H2Aub) by Ring1 subunit, deposing two repressive histone marks on chromatin (Suganuma T et al, 2008). Ezh2, Suz12 and Eed proteins form the core of the PRC2, and the methyltransferase activity of PRC2 requires both Ezh2 and Suz12 subunits (Pasini D et al, 2004). An interaction between RAR and Ezh2 and Suz12 has also been identified (Villa R et al, 2007, Amat R et al, 2011). Polycomb complexes act as global cellular regulators for maintenance of epigenetically repressed states. In embryonic stem (ES) cells PRCs actively repress genes important for embryonic development and cell fate decisions. In untreated stem cells, RA primary target genes, such as the Hoxa1, Cyp26a1, and RARβ2, are targeted by PRCs. Upon RA addition to the stem cells, there is a rapid dissociation of the PRCs from these RA target genes by a mechanism that is not fully understood (Amat R et al, 2011). However, the RA induced PRC2 displacement is not a common feature associated with RA-induced transcription. Laursen KB et al. identified a subset of genes that displayed an increase in PRC2 recruitment simultaneously with transcriptional activation upon RA treatment, functional depletion of the essential PRC2 protein enhanced the RA-associated transcription of these target genes, indicating a role of PRC2 in attenuating their transcription, which entails a delayed induction of these target genes. Thus in addition to maintaining transcriptional repression, the PRC2 complex is able to attenuate the transcriptional activation of specific genes during stem cell differentiation. Such a mechanism would permit the fine-tuning of transcriptional networks during RA induced differentiation (Laursen KB et al, 2013). However, how PRC2 complex is recruited to target these specific genes still awaits further studies.

Some other examples of the epigenetic changes that occur in response to RA and their relationship to cell differentiation have been documented. For example, a histone demethylase PHF8 was shown to be involved in the response of stem cell to RA. An RA induced interaction between PHF8 and RAR was identified, and PHF8 has been reported to act as an RAR co-activator depending on its activities for both H3K4me3/2/1 and H3K9me2/1 demethylation, and PHF8 knockdown in P19 teratocarcinoma stem cells results in reduced RA-induced neural differentiation (Qiu J et al, 2010). Conversely, SETD6 a lysine methyltransferase, which monomethylates H2AZ on lysine 7 and is required for the maintenance of embryonic stem cell self-renewal. RA treatment rapidly removes these monomethyl marks, and similarly, a genetic reduction in SETD6 expression in ES cells results in differentiation and loss of self-renewal ability (Binda O et al, 2013). Similarly, a secondary coactivator for RAR, CARM1, which catalzes H3R17, 26 methylation was shown involved in SRC3 mediated activation of Nanog expression, suggesting a role of CARM1 in pluripotency maintenance (Wu Z et al, 2012), in support of this function, depletion of CARM1 in embryonic stem (ES) cells results in loss of pluripotency and induction of differentiation (Wu Q et al, 2009).

PML-RAR is an oncogenic transcription factor found in acute promyelocytic leukemias (APLs), it derives from a 15;17 chromosomal translocation, which fuses the gene encoding for the RARα located on chromosome 17 to the promyelocytic leukemia (PML) gene on chromosome 15 (Chen J et al, 2010). PML-RAR functions as a transcriptional regulator of RA target genes. DNA methylation was shown to have an important role in PML-RARα mediated blocking of differentiation of hematopoietic precursors and APL leukemogenesis. Studies showed that PML-RAR induces silencing of RA signaling pathways by recruiting DNMTs (DNA methyltransferases) to target promoters, such as a putative tumor suppressor gene RARβ2, and the hypermethylation contributes to its leukemogenic potential (Di Croce L et al, 2002). At pharmacological doses, RA overcomes this repression, probably by reducing DNMTs expression and activity, resulting in terminal differentiation and reversion of the transformed phenotype (Fazi F et al, 2005). It is interesting to investigate whether the active DNA demethylation initiated by TET family proteins (refer to TET proteins part) are also involved in overcoming the repressive activity of PML-RAR.

Collectively, RA induces differentiation primarily through its receptor RARs. RA, through binding to the RARs, alters interactions of the RARs with coregulatory proteins at numerous genes in stem cells, and some of these coregulators are epigenetic modifiers, which then either place or remove epigenetic marks on histones or DNA, altering chromatin structure and leading to an exit from the self-renewing, pluripotent stem cell state.

3.2 Cell Models to Study RA Induced Stem Cell Differentiation

Among the various RA sensitive tissues and cell types, embryonal carcinoma (EC) and later, embryonic stem (ES) cells were shown to undergo differentiation upon RA stimulation (Mummery CL et al, 1990). ES and EC cells are characterized by their self-renewal capacity as well as their ability to differentiate into various cell lineages, furthermore, they are very amenable to genetic manipulations, making them

excellent model systems to address fundamental mechanistic questions during RA-induced differentiation, and also providing invaluable biological models to study early developmental processes.

EC cells are self-renewing and pluripotent in most cases, they lack the ability when reintroduced into the developing embryo to participate in embryogenesis and give rise to a wide variety of tissues. One of the most commonly used EC cell lines for the study of RA-dependent differentiation is F9 cells. F9 can differentiate into one of the three extraembryonic endoderms (i.e., primitive, parietal, and visceral), depending on the culture conditions: treatment of F9 cells grown in monolayer culture with RA results in differentiation to primitive endoderm, while treatment with both RA and cAMP causes differentiation to parietal endoderm; in addition, treatment of F9 cells grown as aggregates induces differentiation to visceral endoderm (Gudas, 1991; Strickland and Mahdavi, 1978; Strickland et al., 1980). Unlike EC cells, ES cells are diploid, participate in embryogenesis, and are able to differentiate into germ cells in vivo. RA can induce differentiation of ES cells into a large number of different cell types including neurons, glial cells, adipocytes, chondrocytes, osteocytes, corneal epithelium, skeletal muscle, smooth muscle, and ventricular cardiomyocytes depending on both the timing of RA treatment and the addition of other factors (Eiges and Benvenisty, 2002; Rohwedel et al., 1999; Schuldiner et al., 2000). In addition, though ES and F9 cells differentiate into different cell lineages upon RA treatment, studies showed that ES and F9 cells showed similar RA response at the early stage of differentiation (Mahony S et al, 2011; Gerard Benoit et al, 2015). All these features make ES and F9 cells good cellular model systems, which can mimic the physiological processes occurring during embryogenesis, to improve our understanding on the molecular mechanisms governing RA induced embryonic development.

4 DNA Methylation

One of the best characterized epigenetic modifications in mammalian genomes is DNA methylation. It is involved in different key cellular processes, including X-chromosome inactivation, imprinting and transcriptional silencing of specific genes and repetitive elements. DNA methylation is a biochemical process during which a methyl group was transferred to cytosine within CpG dimucleotides, generating 5-methylcytosine (5mC; Goll & Bestor, 2005). This process is catalyzed by a group of DNA methyltransferases (DNMTs), consisting of DNMT3a, DNMT3b and DNMT1. DNMT3a and DNMT3b are de novo methyltransferases that establish DNA methylation patterns by oxidizing unmethylated CpG sites (Okano M et al, 1998,1999). DNMT1 plays a key role in the maintenance of established DNA methylation patterns, it localizes to replication foci during S-phase, where it preferentially methylates hemi-methylated CpGs with the help of UHRF1 (Bostick et al, 2007).

DNA methylation has been associated with tumorigenesis as most cancer cells display aberrant DNA methylation patterns (reviewed in Jones & Baylin, 2007). This includes both global hypomethylation of the genome and promoter-specific hypermethylation. Global hypomethylation renders genome unstable and local hypermethylation leads repression of tumour suppressor genes needed to fight cancer (You & Jones, 2012).

4.1 Genome-Wide Reprogramming of DNA Methylation Pattern

Genomic methylation patterns in somatic differentiated cells are generally stable and heritable. However, the mammalian genome undergoes genome wide reprograming of DNA methylation patterns during early embryogenesis and gametogenesis (Figure 17). Upon ferterlization, the paternal genome undergoes rapid active genome-wide demethylation, while the maternal genome exhibits a gradual loss of methylation until the eight-cell stage. Methylation re-establishment occurs at around implantation for both genomes. During the process of gametogenesis, primordial germ cells(PGCs) become demethylated early in development, While methylation is re-established in prenatal male germ cells, and after birth in growing oocytes (Reik W et al, 2001). Collectively, global DNA demethylation is important for setting up pluripotent states in early embryos and for erasing parental-origin-specific imprints in developing PGCs (Feng S et al,2010).

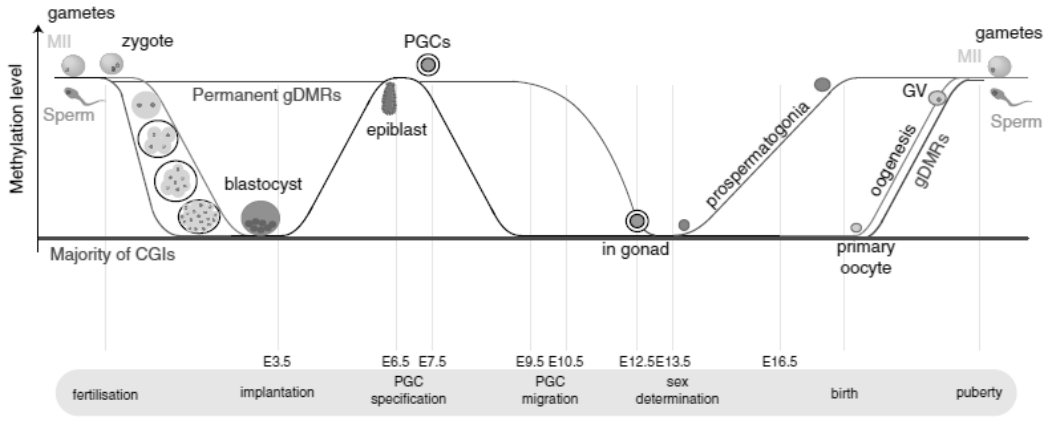


Figure 17 Methylation reprogramming in preimplantation embryos and germ lines (Saadeh H et al, 2014).

Upon fertilization genome-wide DNA demethylation occurs in the zygote, with active demethylation for the paternal genome and a gradual passive DNA demethylation for maternal genome, both are remethylated around the time of implantation; In the germ line, PGCs initiate a phase of comprehensive DNA demethylation, which enables subsequent establishment of a unique gamete-specific methylome during gametogenesis. The paternal genome is shown in blue while the maternal genome is shown in red. The black line represents both parental genomes. PGCs, primordial germ cells; GV, germinal vesicle oocyte; MII, metaphase II oocyte; E, embryonic day.

4.2 Role of DNA Methylation in Transcriptional Regulation

4.2.1 Correlation between DNA Methylation and Gene Expression Silencing

DNA methylation is most frequently found at CpG dinucleotides, and although rare non-CpG methylation occurs, its function remains unclear (Lister et al, 2009). CpG dinucleotides are found unevenly distributed throughout the genome. Certain regions with a high density of CpG and are referred to as CpG islands (CGI). Global methylomes showed that “single” CpGs are generally hypermethylated, while CpG islands remain predominantly hypomethylated (Deaton and Bird 2011). CGIs are often associated with gene promoters, particularly those of developmental or housekeeping genes, where they stay hypomethylated most of the cases (Saxonov S et al, 2006). The hypomethylated

state of promoter CGIs results from binding of transcriptional factors or presence of active histone modification markers (like H3K4me3), which inhibit the DNMTs recruitment to those target regions (Meissner 2011 ;Erfurth et al, 2008; Thomson et al, 2010).

Historically, DNA methylation at promoter regions has been linked to transcriptional repression. However, detailed analysis of CGI-associated promoters and their association with target gene expression revealed that different gene promoters respond differently to DNA methylation according to their CpG content and chromatin micro-environment. Gene promoters could be classified into three categories according to their CpG density: Low CpG density promoter (LCP); intermediate CpG density promoter (ICP) and high CpG density promoter (HCP) (Figure.18).

Genes with low CpG density promoters (LCPs) are usually transcriptionally active regardless of their methylation state (Meissner et al, 2008). Intermediate CpG density promoters (ICPs) are inactive when methylated (Meissner et al, 2008), which supports the traditional view that DNA methylation represses transcription. However, hypermethylation of ICPs occurs more frequently during differentiation, such as at pluripotency gene promoters, where DNA methylation is thought to reinforce the silencing during differentiation (Meissner et al, 2008; Borgel et al, 2010). High CpG density promoters (HCPs) are associated with housekeeping genes and some key developmental genes. HCPs are rarely DNA methylated (Weber et al, 2007; Meissner et al, 2008), however, the hypomethylation state of HCPs is not always correlated with transcriptional activation. HCPs at housekeeping genes are enriched with the transcription initiation mark H3K4me3 and are generally highly expressed, whereas those at developmental genes are enriched with both H3K4me3 and the repressive mark H3K27me3 and are generally silent (Meissner et al, 2008).

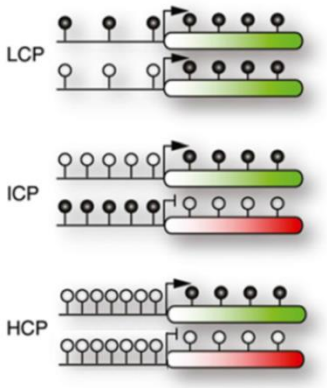


Figure 18 Three categories of gene promoter respond differently to DNA methylation.

Gene promoters are categorized into three types according to CpG density: (LCP) Low CpG density promoter; (ICP) intermediate CpG density promoter; (HCP) high CpG density promoter. They respond differently to methylation, methylation on LCPs fail to repress gene expression, while methylation of ICPs results in efficient gene silencing. HCPs are usually hypomethylated, and it is correlated to gene activation or repression depending on the local

chromatin environment. White cycles represent unmethylated cytosines, black cycles represent methylated cytosines.

Distal regulatory regions such as enhancers are often required to establish correct gene expression patterns in mammalian cells (West AG et al, 2005). Transcriptional enhancers support tissue-specific expression profiles through physical interactions with gene promoters. Enhancers display a unique pattern of DNA methylation (stadler et al, 2011). Methylation of enhancers is closely related to gene expression levels across the genome, and hypermethylation is correlated with repressed gene expression (Aran et al, 2013). Moreover, altered methylation of enhancer sites is more closely related to gene dysregulation in cancer than that of promoter methylation (Aran et al, 2013).

4.2.2 Mechanisms Coupling DNA Methylation to Gene Expression Silencing

DNA methylation is usually linked with transcriptional silencing of associated genes (Kass SU et al, 1997; Siegfried Z et al, 1999), and much effort has been invested in studying the mechanisms that underpin this relationship.

Several working models have been proposed. In some instances, cytosine methylation could directly repress transcription by physically blocking the binding of a transcription factor (TF) to its target sequence (Watt F et al, 1988). For some promoters, repression mediated by DNA methylation is more efficient in a chromatin context, indicating that the surrounding chromatin modification might be involved in this repression effect (Buschhausen G et al, 1987). In consistent with this observation, 5mC can prevent chromatin remodelers that possess CXXC domains from binding to DNA (Lee et al, 2001). These remodelers include H3K4 methyltransferase mixed-lineage leukaemia (MLL) and Lys-specific demethylase 2A (KDM2A), which removes H3K36 methylation marks (Blackledge et al, 2010; Allen et al, 2006). The reader protein for 5mC, such as Methyl CpG-binding protein (MBD), could also help to mediate gene repression by recruiting histone deacetylases or repressive histone H3 Lys9 methyltransferases (H3K9MTs) (Ng HH et al, 2000; Sarraf SA et al, 2004). DNA methyltransferases can be recruited to set up the silenced state by depositing methylated cytosine. In addition to this catalytic role, studies showed that DNMTs have a non-enzymatic role in transcriptional silencing. DNA methyltransferases (DNMTs), including DNMT1, recruit histone deacetylases (HDACs) (Fuks F et al, 2000,2001 ; Geiman et al, 2004) and H3K9 methyltransferases to target DNA (Fuks F et al, 2003), leading to target gene repression by favoring a compacted chromatin landscape. Moreover, studies showed that methylation within the gene body have a dampening effect on transcriptional elongation, possibly by interfering RNA Pol II binding (Lorincz MC et al, 2004).

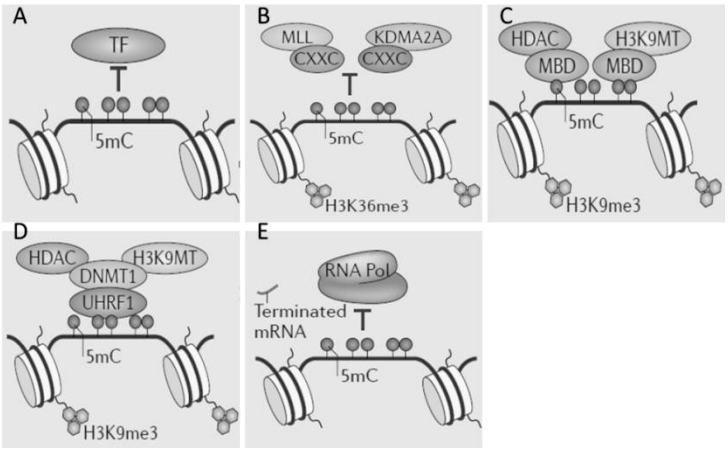


Figure 19 Mechanisms of DNA-methylation-mediated repression (modified from Pastor WA et al, 2013). (A)DNA methylation could silence target gene expression by blocking transcriptional factors (TFs) from binding to cognate DNA sequence. (B) 5mC prevents the binding of H3K4 methyltransferase MLL (mixed-lineage leukaemia) and KDMA2A (Lys-specific demethylase 2A). MLL contribute gene activation by depositing active H3K4 methylation, while KDMA2A facilitate gene expression by removing repressive H3K36me3 mark. (C) Methyl-CpG-binding proteins (MBPs) directly recognize methylated DNA and recruit co-repressor molecules, such as HDACs and repressive histone H3 Lys9 methyltransferases (H3K9MTs), to modify surrounding chromatin and to silence transcription. (D) DNMT enzymes are also physically interact with histone deacetylase (HDAC) and histone methyltransferase (HMT), thus the addition of methyl groups to DNA by DNMTs is coupled to chromatin modification, concurrently leading to gene silencing. (E) DNA methylation within gene body prevents DNA binding of RNA Pol II, thereby to interfere transcriptional elongation.

5 TET Family Proteins

DNA methylation pattern undergoes two major waves of reprogramming during development, and the rapid DNA demethylation during these reprogramming process could not be fully explained by replication dependent passive loss of 5mC, suggesting the existing of enzymes catalyzing the active demethylation. In 2009, the characterization of Ten Eleven Translocation (TET) enzymes, which oxidize methylated cytosine to hydroxymethylcytosine (Kriaucionis & Heintz, 2009; Tahiliani et al, 2009), shed light on the mechanisms underline the active demethylation process.

5.1 Mechanisms of TET-mediated DNA Demethylation

Identified as DNA hydroxylases, TET proteins can catalyze iterative oxidation of methylated cytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani et al, 2009; Ito et al., 2011; He et al., 2011). Interest in TET proteins was primarily centred around the possibility that these oxidized methylcytosines could serve as intermediates within DNA demethylation pathways. Through their catalytic activity, the TETs act as initiators of DNA demethylation, there are at least four mechanisms by which TET proteins could mediate DNA demethylation (Figure.20).

5.1.1 Passive DNA Demethylation Coupled with DNA Replication

After the establishment of DNA methylation pattern by de novo DNA methyltransferase DNMT3a and DNMT3b, this epigenetic feature is maintained through cell division by DNMT1 and its obligate partner UHRF1. The underline mechanism for DNA methylation maintenance is well studied: DNA replication yields two strands with hemimethylated CpG sites, UHRF1 recognizes hemimethylated CpG sites via its SAD/ SRA (SET associated Deinococcus domain (SAD)/SET and RING associated (SRA) domain) domain and recruits DNMT1, then the methylation of the CpG sites on the nascent DNA strand is accomplished by DNMT1 (Bostick M et al, 2007).

However, the affinity between UHRF1 and hemimethylated CpG sites is severely impaired by the oxidation of the methyl group on the cytosine within CpG dinucleotides. The in vitro binding assay shows that UHRF1–hemi-5hmC binding is tenfold less efficient than UHRF1-hemi-5mC binding (Hashimoto H et al, 2012); moreover, it is shown that the enzyme activity of DNMT1 is also reduced towards hemi-5hmC in vitro (Hashimoto H et al, 2012;Valinluck V et al, 2007). All these results imply that the TET-mediated hydroxymethylation of a methylated CpG site in vivo can block methylation maintenance by interfering the recruitment and/or the intrinsic activity of DNMT1, thus promote the dilution of DNA methylation level in a DNA replication-dependent manner.

Although rendering efficient global DNA demethylation, this mechanism does not allow non-global, locus-specific removal of DNA methylation marks. Furthermore, the passive mechanism cannot account for the rapid DNA demethylation in slowly or non-dividing cells.

5.1.2 Active DNA Demethylation Coupled with DNA Repair

TETs catalyzed production of 5hmC could be an intermediate in an active demethylation pathway that ultimately replaces 5mC with cytosine in non-dividing cells. These active mechanisms have been proposed to couple the methylcytosine oxidase activity of TET proteins with specific DNA repair mechanisms. TET proteins were shown to be able to further catalyze the iterative oxidation of 5hmC to 5fC and 5caC, these intermediates could be replaced by unmodified cytosine with the help of TDG/BER mediated base excision repair (He et al, 2011; Ito et al, 2011; Pfaffeneder et al, 2011). In consistent with this model, depletion of TDG causes an increase in the levels of 5fC and 5caC in ES cells (Song CX et al, 2013; Shen L et al, 2013). Another mechanism involved AID (activation-induced cytidine deaminase) and APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) was also proposed. In this model, 5hmC is sensitive to AID and APOBEC family enzymes catalyzed deamination, and the deamination product, 5-hydroxymethyluracil (5hmU), was further removed by SMUG1 (single-strand-selective monofunctional uracil DNA glycosylase) or TDG coupled with base-excision repair (BER) pathway (Guo JU et al, 2011; Nabel CS et al,2012).

5.1.3 Decarboxylation of 5caC by Unknown Enzymes

Genome-wide analysis referred to the distribution of the intermediates, 5hmC, 5fC and 5caC, during DNA demethylation process revealed a much less abundance than 5hmC in the investigated cells and tissues even in a TDG-deficient cell (Shen L et al, 2013), suggesting a existence of decarboxylases for 5caC or deformylases for 5fC. Schiesser S et al provided evidences for the existence of decarboxylases.

They incubated a N^{15} labelled oligonucleotide containing 5caC with ES cell lysates, and a decreased signal was detected afterwards, indicating direct conversion of 5caC to cytosine without BER (Schiesser S et al, 2012). However, the enzyme responsible for this process remains to be identified.

5.1.4 Involvement of DNMT Enzymes

Interestingly, the DNMTs have also been suggested to be involved in DNA demethylation process by directly converting 5hmC to unmethylated cytosine. Chen et al showed that DNMT3a and DNMT3b, but not the maintenance enzyme DNMT1, could serve as DNA dehydroxymethylases to directly convert 5-hmC to unmethylated cytosine. Moreover, the intactness of the methylation catalytic sites is also required for their 5-hmC dehydroxymethylation activity. Function bidirectionally as DNA methyltransferases and as dehydroxymethylases, reducing conditions favour the methyltransferase activity of DNMT3a/DNMT3b, whereas oxidizing conditions favour their dehydroxymethylation (Chen CC et al, 2012). Since all the results supporting this hypothesis were got from *in vitro* data, whether this reaction occurs *in vivo* is still unknown.

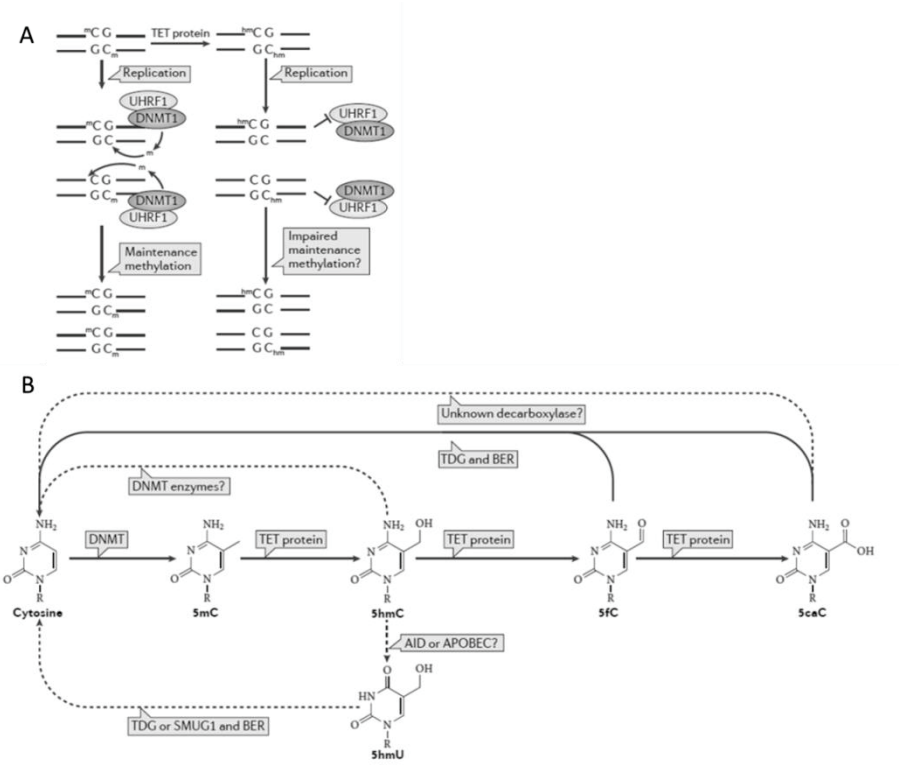


Figure 20 Potential mechanisms of TET proteins mediated DNA demethylation.

(A)Mechanism of passive DNA demethylation coupled with DNA replication. DNA strands were asymmetrically methylated after DNA replication, DNMT1 is recruited to the hemi-methylated DNA by UHRF1, and DNMT1 then restores symmetrical methylation pattern. The oxidative cytosine modification catalyzed by TET proteins may impair maintenance methylation by inhibiting UHRF1 binding and intrinsic DNMT1 activity, thereby to facilitate the dilution of modified DNA during DNA replication. (B) Other putative mechanisms of DNA demethylation. TET

proteins catalyze iterative oxidation of 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine), 5fC (5-formylcytosine) and 5caC (5-carboxylcytosine). 5fC and 5caC can be removed by TDG/BER mediated base excision repair; alternatively, 5hmC was also proposed to undergo deamination by AID/APOBEC (activation-induced deaminase/ apolipoprotein B mRNA editing enzyme), the 5hmU could be further converted to cytosine with the help of SMUG1 (single-strand-selective monofunctional uracil DNA glycosylase) or TDG and BER pathway. It remains unknown whether there are decarboxylases or deformylases that can remove the modification directly. And *in vitro* data suggested an involvement of DNMTs in directly dehydroxylazing 5hmC to cytosine.

5.2 Domain Structure of TET Family Proteins

There are three members in TET proteins family: TET1, TET2 and TET3. The three proteins share a high degree of homology within their C-terminal catalytic domain, which consists of a double strand β-helix (DSBH) domain and a preceding cysteine (Cys) rich domain. The catalytic domains of TET proteins are characteristic of Fe2+ and 2-oxoglutarate (2OG)-dependent dioxygenases, and all three TETs can iteratively oxidize 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Tahiliani et al, 2009; Ito et al., 2011; He et al., 2011). The DSBH fold, which comprises the catalytic domain of all TETs, contains an His-X-Asp/Glu signature motif, a C-terminal conserved His residue and Arg residue that is involved in coordinating Fe2+ or 2-oxoglutarate respectively (Loenarz C et al., 2011). A crystal structure of human TET2-DNA complex was shown in Figure. 21. The structure shows that two zinc fingers bring the Cys-rich and DSBH domains together to form a compact catalytic domain. The Cys-rich domain stabilizes the DNA above the DSBH core. Moreover, the negatively charged DNA binds the basic amino acids located at the surface of the TET2 and that the enzyme flips the methylcytosine out of the DNA double helix into its double-stranded b-helix catalytic pocket where it is stabilized by hydrogen bonds (Hu L et al., 2013).

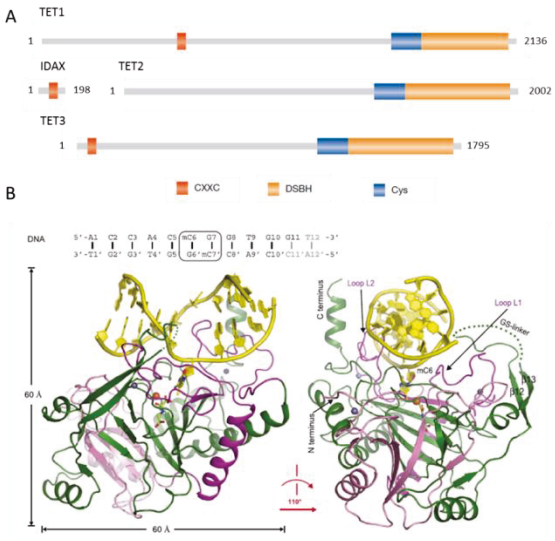


Figure 21 Domain structure of TET1-3 and crystal structure of TET2-DNA complex.
(A)TET1–3 contain a cysteine (Cys)-rich region followed by the double stranded β-helix (DSBH) fold characteristic of the 2OG-Fe(II) oxygenases and required for catalytic activity. TET1 and TET3 also contain a CXXC domain towards

the amino terminus, a chromosomal inversion detached the catalytic domain of TET2 from its CXXC domain, which became a separate gene, which encodes IDAX (inhibition of the Dvl and axin complex). The number of amino acids of human region is indicated. (B) A cartoon representation of TET2catalytic domain-DNA structure in two different views. The Cys-rich domain is indicated in purple while the DSBH domain is indicated in gree, DNA is colored in yellow, an Fe(II) iron and three zinc cations are shown as red and gray balls, respectively. The methylated cytosine is shown to be inserted into the catalytic cavity with the methyl group orientated to catalytic Fe(II) for reaction.

Another distinct feature of TET family proteins is the CXXC zinc finger domain. A CXXC domain can be found at the N terminal of TET1 and TET3. The CXXC domain of TET2 is reported to be detached from the TET2 gene through a chromosomal invert event during evolution, and became a separate gene that encodes IDAX (inhibition of the Dvl and axin complex; also known as CXXC4) (Iyer LM et al, 2009). The CXXC domain was found in many other chromatin-associated proteins, such as DNMT1 and myeloid/lymphoid or mixed-lineage leukemia (MLL), which were shown to specifically recognize unmodified CG sequences and to target proteins to CpG-rich sequences in the genome (Song J et al, 2011; Cierpicki T et al, 2010).

The CXXC domain of TET1 recognizes not only unmodified cytosine but also 5mC and 5hmC, with a preference for genomic regions of high CpG content (Zhang et al., 2010; Xu Y et al., 2011). Consistent with this feature, ChIP-seq approaches revealed an enrichment of TET1 around transcription start sites (TSSs) in mouse ES cells (Xu Y et al., 2011; Williams et al., 2011; Wu et al., 2011). The IDAX CXXC domain showed a preference for unmethylated CpG sequences in vitro, and genome-wide distribution of IDAX revealed that IDAX binds preferentially to hypomethylated CpG islands and CpG-rich promoters in cells. Interestingly, IDAX is shown to target TET2 for destruction via a caspase-dependent mechanism, in a manner that depends on DNA binding through the IDAX CXXC domain, suggesting that IDAX recruits TET2 to DNA before degrading it (Ko M et al, 2013). The TET3 CXXC domain can target unmethylated cytosines within both CpG and non-CpG contexts (Xu Y et al, 2012). Moreover, the CXXC domain of TET3 was also shown to autoregulates its protein levels via caspase activation, moreover, the catalytic activity of TET3 was also shown to be negatively regulated by its CXXC domain, perhaps through an autoinhibitory mechanism that involves a physical interaction between the CXXC and catalytic domains or, alternatively, by tethering TET3 to specific DNA binding sites and thus limiting its genome-wide activity (Ko M et al, 2013). Thus, unlike other CXXC domains that specifically recognize unmethylated CpG sites, CXXC domains of TET1/3 and IDAX were shown to have increased flexibility in sequence selectivity and might play a role in facilitating the recruitment of TET proteins to their specific genomic targets.

5.3 Roles of TET Proteins in Transcriptional Regulation

5.3.1 Dioxygenase Activity Dependent Roles of TETs in Transcriptional Regulation

5.3.1.1 Correlation Between Oxidized Methylcytosines and Gene Expression

Given the important role of DNA methylation in transcriptional regulation (refer to DNA methylation part), with the characterization of the enzymatic activity of TET proteins, it was anticipated that they

would regulate transcription by adjusting DNA methylation levels. Since the description of TET enzyme activity, much effort has been invested in exploring the genome-wide distribution of oxidized mthylcytosines (5hmC, 5fC and 5caC), this genome-wide mapping of oxidized 5mC bases in the mammalian genome represents an unbiased approach to investigate the potential oxygenase activity dependent role of TETs in transcriptional regulation.

Oxidized Methylcytosines at Gene Promoters

As we discussed in DNA methylation part, gene promoters can be divided into high CpG denstity promoters (HCPs), intermediate CpG density promoters (ICPs) and Low CpG density promoters (LCPs). As methylation on cytosine is the prerequisite for subsequent oxidation of the methyl group, the absence of 5mC at HCPs predicts a minimal presence of oxidized form of methyl-cytosine at these promoters. Consistently, genome-wide mapping studies showed that HCPs are generally devoid of 5hmC/5fC/5caC in mouse ESCs (Shen L et al, 2013). Moreover, it is also showed that 5hmC is preferentially enriched at promoters of genes expressed at medium-to-low levels in mouse and human ESCs (Pastor et al, 2011; Szulwach et al, 2011), and the promoters of these genes are generally associated with ICPs and LCPs. Studies from Yu et al. also indicate that that 5hmC is more abundant at ICPs/LCPs than at HCPs (Yu et al, 2012). In ES cells, a similar distribution pattern for 5fC was found for 5hmC (Raiber et al, 2012). Recent studies showed that 5fC/5caC tend to accumulate at these promoters upon TDG depletion (Song et al, 2013; Shen et al, 2013), indicating that TET/TDG-mediated demethylation occurs at these loci.

Oxidized methylcytosines within gene bodies

Enrichment of 5hmC within gene bodies, especially the 3 prime of gene bodies, is found in all cell type investigated, including human and mouse ESCs, mouse liver and brain (Pastor et al, 2011; Szulwach et al, 2011; Hahn et al, 2013). Besides, hydroxymethylation within gene bodies is positively correlated with gene expression (Wu H et al, 2011). Although 5fC and 5caC are much less present in the genome, 0.2–0.3% of the total methylcytosines for 5fC and much less for 5caC (Shen et al, 2013), they also exhibit a tendency to accumulate within actively transcribed gene bodies upon TDG depletion (Song et al, 2013; Shen et al, 2013). The exact mechanisms underline this positive correlation is still elusive, one possible function of intragenic 5hmC/5fC/ 5caC is to regulate the binding of RNA polymerase II during transcriptional initiation or elongation (Kellinger et al, 2012 ; Thalhammer et al, 2011).

Oxidized methylcytosines at enhancers

Different genome-wide mapping studies indicate that 5hmC is highly enriched at active enhancers (Yu et al, 2012; Stadler et al, 2011; Szulwach et al, 2011). However, 5hmC is found enriched immediately adjacent to, instead of the precise transcription factor-binding sites (Yu et al, 2012). According to this distribution pattern of 5hmC, it is still unknown but interesting to investigate whether 5hmC facilitates the binding of transcriptional factors or the transcriptional factors recruit TETs to the binding sites or whether both mechanisms operate and reinforce one another. Moreover, the reduction in 5hmC level at transcription factor-binding sites might result from TETs-mediated hydroxylation of 5mC, but could also due to the blocked binding site for DNMTs by transcription factors. Furthermore, 5fC and 5caC are also

enriched at enhancers in ES cells upon TDG deletion, consistent with TET-mediated demethylation of enhancers (Song et al, 2013; Shen et al, 2013). Further studies will be necessary to determine the extent to which TETs-mediated demethylation contribute to function of enhancers.

5.3.1.2 Oxygenase Activity Dependent Role of TET1 in Transcriptional Regulation.

To gain more insight into the functional role of TETs in transcriptional regulation, genome-wide occupancies of TET proteins in ESCs, HEK293T cells and bone marrow tissues have been investigated. All three TETs proteins showed preferential localization to unmethylated CpG-rich promoters, this preference is probably due to the N-terminal CXXC domain, which has a high affinity for unmethylated CpG dinucleotides (Chen et al, 2013; Deplus et al, 2013; Vella et al, 2013; Williams et al, 2011; Wu et al, 2011c).

Genome-wide mapping of TET1 binding in ESCs showed that TET1 mainly binds to gene-rich regions, with the highest preference for transcription start sites (TSSs) and less intense binding throughout gene bodies (Williams et al, 2011; Wu et al, 2011; Xu et al, 2011). Based on the genome-wide study of 5hmC and TET1 in ESCs, overlay of 5hmC-positive regions and TET1 binding demonstrated a significant, although not complete, overlap both genome-wide and at promoters. Depletion of TET1 led to a significant decrease in 5hmC levels, indicating the dependency of 5hmC signal on TET1 expression (Williams et al, 2011; Wu et al, 2011b; Xu et al, 2011).

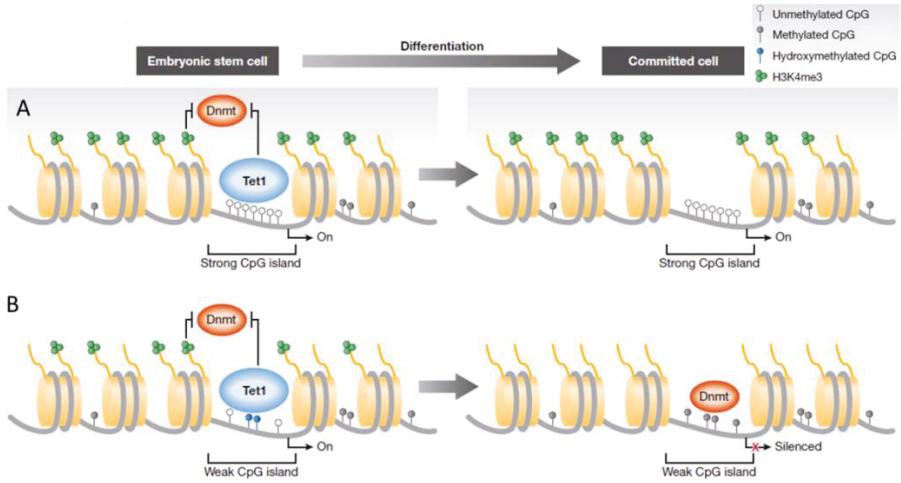


Figure 22 Potential role of TET1 in transcriptional activation (Williams K et al, 2011).
TET1 might contribute to transcriptional activation by preventing DNA methylation. TET1 is found enriched at both CpG rich promoters (A) and also weak CpG islands (B), TET1 binding helps to maintain hypomethylated state at the promoters, it might also act as a failsafe mechanism to remove aberrant DNA methylation. Moreover, TET1 binding might also have a role in ensuring the timely methylation and silencing of these target genes during differentiation.

The CpG-rich promoters are mainly unmethylated in ESCs, normally, it is thought to be protected from DNA methylation by high levels of H3K4me3 that inhibit the recruitment of de novo DNMTs, the enrichment of TET1 on these promoters suggests a role of TET1 in maintaining CpG islands free of methylation. In support of this model, TET1-bound CGIs are generally hypomethylated, and depletion of

TET1 in mouse ESCs allows for DNA methylation to occur at many CGIs (Wu et al, 2011). In addition to the enrichment at CpG-rich promoters, TET1 is also shown to bind a subset of actively transcribed CpG-poor gene promoters, such as promoters for pluripotency-associated factors (Wu et al, 2011). These promoters have been reported to become de novo DNA-methylated during differentiation (Mohn F et al, 2008). At these promoters, TET1 serves as a guardian protein to maintain their transcriptionally active state by preventing DNA methylation in undifferentiated ESCs, and to ensure the timely methylation and silencing of these target genes during differentiation.

5.3.2 Dioxygenase Activity Independent Roles of TETs in Transcriptional Regulation

Although many studies have indicated that the enzymatic activity of the TET family proteins is important for their function in transcriptional regulation, accumulating evidences suggested that TET proteins, might also exert functions independently of their catalytic activity (Williams et al, 2011; Deplus R et al, 2013; Yildirim et al, 2011). Williams et al. found that the tested transcriptional effects by knockdown of TET1 were similar in normal and DNMT TKO ES cells (in which both 5mC and 5hmC modifications are absent), suggesting that the effects are independent of catalytic activity. The search for TET interactants has brought light to their catalytic activity independent role in transcriptional regulation. TET proteins are shown to serve as scaffolding proteins recruiting other proteins which have a role in shaping chromatin landscape by favoring an open or closed chromatin state (Figure.23). These interactants include chromatin-associated proteins involved in transcriptional activation (OGT and SET1/COMPASS complex) or repression (SIN3A/HDACs, NURD) (Delatte B et al, 2014). Moreover, genome-wide studies have shown that TET1 is associated not only with active promoters (marked with “H3K4me3 only”) but also with bivalent poised promoters (marked with “H3K4me3 and H3K27me3”) and repressed promoters (marked with “H3K27me3 only”). Depletion of TET1 causes transcriptional activation and repression of many direct TET1 targets (Williams et al, 2011; Wu H et al, 2011; Xu, Y et al, 2011), suggesting that TET proteins, more versatile than anticipated, may have dual functions in transcriptional regulation via distinct mechanisms. Below I will describe in more details about how these transcription-related factors help TETs to perform their functions.

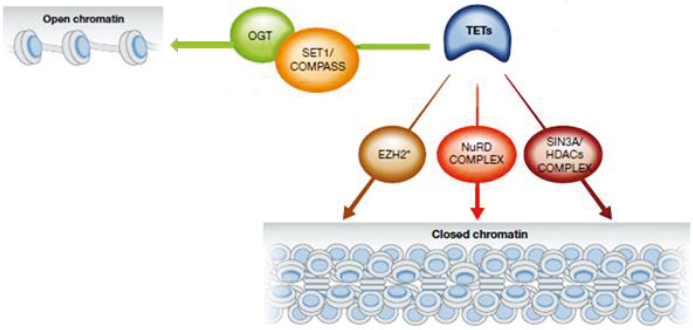


Figure 23 Interaction partners help TETs to regulate transcription (Delatte B et al, 2014).

TET proteins serve as scaffolding proteins recruiting several proteins and catalytically active complex, which have a role in shaping the chromatin landscape by favoring an open or closed chromatin state. TETs-OGT/SET1/COMPASS interaction help to open the chromatin, thereby to facilitate gene activation; while other partners, such as EZH2, NuRD complex and Sin3A complex, are involved in transcriptional repression by condensing the surrounding chromatin.

5.3.2.1 Dioxygenase Activity Independent Roles of TETs in Transcriptional Activation

The transcriptional activation role of TET1 may be partially mediated dependent on its 5mC oxidation activity to maintain unmethylated states at active promoters or distal enhancers (as discussed in Figure.22). A physical interaction partner for TET proteins (TET2 and TET3, to a lesser extent for TET1)—OGT (O-linked β-N-acetylglucosamine (O-GlcNAc) transferase)—is also shown to be involved in TET proteins mediated transcriptional activation (Shi et al, 2013; Vella et al, 2013; Ito et al, 2014).

OGT is a glycosyltransferase, it adds a GlcNAc sugar to Ser and Thr residues of numerous proteins, including histone H2B and other chromatin modifiers (Hu et al, 2010). TET proteins are shown to recruit OGT to promoters, depletion of TET proteins diminishes the association of OGT with chromatin (Chen et al, 2013; Vella P et al, 2013), in consistent with this, ChIp-seq analysis showed a co-localization of TETs and OGT within genome (Deplus R et al, 2013). Various mechanisms by which OGT could influence transcription of TETs target genes were proposed. Firstly, OGT could activate TETs target genes expression by catalyzing O-GlcNAcylation of serine 112 of H2B (H2BS112GlcNAc), H2B S112 GlcNAcylation then promotes mono-ubiquitination of lysine 120 (H2BK120ub), a histone mark associated with transcriptional activation (Fujiki R et al, 2011). Downregulation of TET2 reduces the amount of H2BSer112GlcNAc marks in vivo, which are associated with gene transcription regulation (Chen Q et al, 2013). Likewise, a component of the H3K4 methyltransferase SET1/COMPASS complex HCF1 (host cell factor 1) is glycosylated by OGT, which is important for the integrity of SET1/COMPASS complex, providing a link between H3K4me3 and TET proteins. A strong decrease of O-GlcNAc and H3K4me3 on TET2 target genes was observed after TET2 depletion (Deplus R et al, 2013), suggesting the presence of a functional complex of TET2/OGT/ SET1/COMPASS on TET2 target genes. These results thus suggest a step-wise model highlighting a novel means by which TETs may induce transcriptional activation (Figure 23). TET-OGT interactions facilitate the recruitment of OGT to chromatin, and enhance its glycosyltransferase activity. This would in turn favors glycosylation of serine 112 of H2B (H2BS112GlcNAc) and ubiquitinylation of lysine 120 (H2BK120ub). In parallel, TETs-OGT mediated stabilization of SET1/COMPASS complex results in its binding to H2BK120ub (Wu M et al, 2008), thus catalyzing the trimethylation of H3K4 (H3K4me3), leading to transcriptional activation (Figure.24).

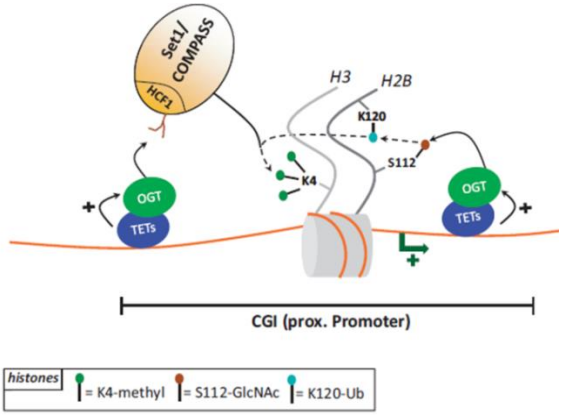


Figure 24 Proposed working model for TETs-OGT-SET1/COMPASS (Delatte B et al, 2013).

TET2 and TET3, to a less extent for TET1, interact with OGT and recruit it to promoter region of TETs target genes, OGT deposits glycosylation at S112 of histone H2B (H2BS112GlcNAc), which in turn favors H2B ubiquitinylation on

lysine 120 (H2BK120ub). On the other hand, OGT glycosylates the Set1/COMPASS complex on its HCF1 subunit, enhancing the complex stability. This would result in Set1/COMPASS binding to H2BK120ub, trimethylation on H3K4 and in transcriptional activation.

5.3.2.2 Dioxygenase Activity Independent Roles of TETs in Transcriptional Repression

The repressive role of TETs in transcriptional regulation is better studied for TET1 in ES cells. Gene expression microarray or RNA-seq analysis of TET1-depleted mouse ES cells revealed that TET1 predominantly has repressive, rather than activating, roles on its direct target genes (Pastor WA et al, 2011; Williams et al., 2011; Wu et al., 2011; Xu et al, 2011). This repression effect might be related to its physical interaction with the SIN3A repressor complex (Williams et al., 2011), or its ability to facilitate the recruitment of the MBD3/NURD (Yildirim et al, 2011) and PRC2 (Wu et al, 2011) to chromatin.

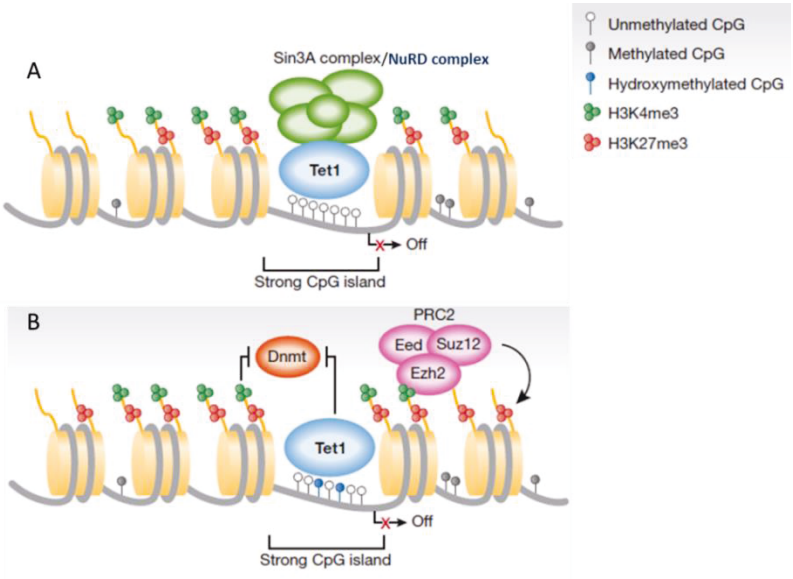


Figure 25 Mechanisms of TET1 mediated transcriptional repression in ESCs (Williams K et al, 2011).
TET1 could be involved in transcriptional repression in ESCs through the recruitment of repressive complexes, which have a role in condensing the chromatin by depositing repressive histone marks on histone tails. (A) TET1 could mediate transcriptional repression by directly recruiting the Sin3A/NuRD co-repressor complex to a subset of its target genes. The HDAC1 and HDAC2 component of these complexes maintain histones in a deacetylated form, thereby to repress transcription. (B) TET1 was also shown to facilitate the recruitment of PRC2 complex to its target genes, leading to transcriptional repression by leaving repressive H3K27me3 marks.

A physical interaction between TET1 and SIN3A was identified, SIN3A is known to associate with histone deacetylases HDAC1 and HDAC2, enzymes that maintain histones in a deacetylated form, thereby to repress transcription. ChIP-seq analysis showed that that SIN3A has a similar binding profile and a significant overlap of target genes as TET1. The recruitment of Sin3A to a subset of these genes was dependent on TET1 expression. Moreover, upon silencing of TET1 or Sin3A, a subset of target genes to which they co-bind are upregulated, indicating that transcriptional repression by TET1 is mediated through the recruitment of Sin3A. Interestingly, the de-repression of the TET1 and Sin3A common target genes were also observed in DNMT TKO ESCs upon TET1 depletion, suggesting that the repressive function of TET1 is independent of its catalytic activity (Williams et al, 2011). Another HDAC1 and HDAC2

containing complex, NuRD complex, was also shown to interact with TET1 in mESCs, and the genome-wide profile of MBD3 NuRD component is similar to that of TET1 (Yildirim et al, 2011; Shi et al, 2013), suggesting a similar working model as that of TET1/SIN3A.

Genome-wide studies indicated an enrichment of TET1 binding at H3K27me3 promoters (Williams et al, 2011), suggesting a role of TET1 in recruiting H3K27me3 "writers" to promoters. In agreement with this, a large subset of TET1-bound promoters is also occupied by PRC2 (Williams et al, 2011; Wu et al, 2011). Although no direct interaction between PRC2 and TET1 was detected, PRC2 binding was found to decrease upon TET1 knockdown (Wu et al, 2011), suggesting that TET1 indirectly facilitate PRC2 chromatin binding.

It thus seems that on repressed promoters (mostly bivalent in mESCs), TET1 might recruit the SIN3A and or NuRD complex (directly) and the PRC2 complex (indirectly), enabling these complexes to maintain a closed chromatin state by catalyzing histone deacetylation and histones H3 deacetylation and K27 trimethylation, therefore acting on gene repression (Figure. 23).

5.3.3 Cross-talk between Dioxygenase Activity Dependent and Independent Role of TETs in Transcriptional Regulation.

As I discussed above, NuRD complex has been found to interact with TET1 in mESCs (Yildirim et al, 2011; Shi et al, 2013), Yildirim and coworkers reported that the repressive NurD complex can bind 5hmC-containing DNA through its MBD3 subunit, suggesting an involvement of TET1 enzyme activity in facilitating NuRD recruitment to promoters. Likewise, since no direct interaction between PRC2 and TET1 was detected, suggesting an indirect facilitation role of TET1 on the recruitment of PRC2 to many TET1 target genes. According to studies indicating that DNA methylation and PRC2 are generally localized at distinct gene promoters in ES cells or cancer cells (Fouse SD et al, 2008; Gal-Yam EN et al, 2008) and high levels of 5mC may inhibit recruitment of PRC2 to chromatin (Bartke T et al, 2010); moreover, genome-wide studies showed a significant enrichment of 5hmC at PRC2 bound bivalent promoters (Pastor et al, 2011), it is plausible that TET1 prepares the binding sites for PRC2 on chromatin, at least in part, by "shaping" the promoter's DNA modification state. Furthermore, interactions between TET proteins and OGT render covalent glycosylation on TETs proteins by OGT (Shi et al, 2013; Vella et al, 2013; Ito et al, 2014). Glycosylation of threonine 535 of TET1 enhances the protein's stability, and depletion of OGT led to reduced TET1 and 5hmC levels on TET1-target genes (Shi et al, 2013), suggesting that TETs-OGT interaction might also have a role in stabilizing TET protein level and its binding at target genes, regulating 5hmC levels.

Collectively, TET proteins could regulate gene expression dependent or independent of its enzyme activity, the two pathways are not mutual exclusive, in contrast, the enzyme activity of TETs could fine-tune the transcriptional regulators related to TETs to perform their role in transcriptional regulation.

Epigenetic genome marking and chromatin regulation are central to establishing gene expression pattern. TET proteins first appeared on the scene as key enzymes involved in regulating DNA methylation dynamics, the challenge is now to uncover the roles they play. With the characterization of

the enzymatic activity of TET proteins, it was anticipated and then confirmed that they would regulate transcription by adjusting levels of DNA methylation at promoters. A new question emitted that to which extent the enzyme activity of TET proteins is involved in their function of regulating gene expression. No direct correlation between genes that were downregulated after TET1 depletion and genes with profound changes in 5hmC and 5mC levels were observed, furthermore, most of the transcriptional activating effects of TET1 were also detected in the DNMT TKO cells (Williams et al, 2011), suggesting that many of these events are independent of TET1 catalytic activity. These results are in agreement with the idea that DNA methylation at promoters help to maintain inactive genes in a silenced state; however, DNA demethylation itself does not lead to gene activation, but rather renders the gene permissive for activation. Another study from Fouse et al. also supports this idea, they showed that in DNMT TKO cells, only a minor fraction of the methylated genes becomes upregulated by the loss of DNA methylation (Fouse et al, 2008). For further studies, the importance of the enzyme activity of TET proteins in their role in transcriptional regulation could be better elucidated with the help of knock-in mutants of TETs (which lost their enzyme activity) in different cell types and during different developmental stages.

In addition to their role in DNA demethylation, the TET proteins may regulate expression independently of their enzymatic activity. As discussed above, TET proteins could serve as scaffolding proteins recruiting other transcriptional regulators to help perform their function. Future studies will be needed to identify additional TET partners, notably partners of TET2 and TET3. The exploration for new interactors will help to shed light on the modes of actions of TET proteins.

Results

General Introduction:

Thyroid hormones (THs) play critical roles in differentiation, growth, and metabolism. The genomic actions of T3 are mediated by thyroid hormone receptors (TRs), which belong to nuclear receptor superfamily. After the identification of TRs in 1980s (Sap J et al, 1986; Thompson CC et al, 1987), the subsequent intensive investigations culminated in the proposal for a canonical pathway for thyroid hormone signaling. The key points are as follows: 1) similar to other members of nuclear receptors, TRs consist of three function domains: the N-terminal A/B domain, a central DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD); 2) TRs can bind DNA as homodimers, or most commonly, as heterodimers with retinoid X receptors (RXRs). These dimers bind mainly DNA at DR4 response elements, made of a direct repeat of two half sites (5'AGGTCA3') separated by 4 nucleotides; 3)The binding of TRs with DNA is independent of ligand treatment: unliganded TRs reside on DNA and represses the basal transcription by recruiting corepressor complexes, exemplified by NCoR and SMRT. The binding of T3 resulted in a conformational change in which the C-terminal helix (helix 12) of TRs repositions, switching TRs binding partners from corepressor complexes to coactivator complexes, such as P160 family members, SWI/SNF chromatin remodeling complex and the mediator complex, contributing to ligand induced transactivation by TRs. By directly recruiting other proteins, or by generating post-translational modifications on histone tails or on DNA, coactivators and corepressors influence the expression of proximal target genes. This general model proposes that epigenetic modifications, including covalent histone tail modifications and DNA methylations, provide essential information for the regulation of gene expression.

The canonical model established 30 years ago is sufficient to explain most of the pleiotropic influences of T3 in physiology and development, and the corresponding pathologies. With the advent of deep DNA sequencing, genome-wide studies related to T3 response genes (RNA-Seq analysis) and TR chromatin occupancy (ChIP-Seq analysis) open a wide field of investigations of T3 signaling. Progresses made over years add complexity to the simplified model, meanwhile, raise new open questions, which are discussed below, to be addressed in future research.

Transcriptome analyses reveal that TRs possess different repertoires of target genes in different cell-types (Chatonnet F et al, 2014). In addition, the response of several well characterized T3 responsive genes actually varies from cells to cells. For example, the neuron-specific RC3/neurogranin regulation by T3 displays regional and temporal selectivity, which is not due to differential distribution of TR (Guadano-Ferraz A et al, 1997; Dowling AL et al, 2000). Moreover, in a given cell-type, genes can display a marked preference for TRα1 or TRβ1 (Winter, H. et al, 2006; Gauthier K et al, 2010; Chatonnet F et al, 2013). Genome wide analyses of TR occupancy (cistrome) provide useful indications on possible mechanisms underline this complication of TR activity. The genome-wide studies performed in neural cells and hepatocytes (Chatonnet F et al, 2013; Ramadoss P et al, 2014; Grontved L et al, 2015) converge to conclude that DR4 elements are predominant over other types of T3 response elements, and that the number of genes with a proximal TR binding largely exceeds the number of T3 responsive genes. Further dissection of TR binding and T3 response in the two different cell types revealed that although the

cistromes largely intersect, the lists of T3 response genes do not overlap in neural cells and liver. In other words, TR binding was observed in both cell types on a subset of cell-type specific regulated genes. More strikingly, there is limited correlation between the occupation of a DR4 by TR and the transactivation of the neighboring gene after T3 treatment. In both cell types, a number of expressed genes with a proximal DR4 occupied by TR do not respond to T3. Therefore, TR binding to proximal sequences is not the only determinant of T3 transactivation. Similarly, the response mediated by TRα1 and TRβ1 in C17.2 is clearly different, and this marked preference for TRα1 or TRβ1 is not correlated with differential chromatin binding of the receptors (Chatonnet F et al, 2013). To conclude, differential binding alone cannot explain why the repertoire of T3 responsive genes differs between hepatocytes and neural cells or between different isoforms in a given cell type. Other parameters, like the expression of different transcription cofactors in different cell types, must take part in cell-specific or receptor-selective response. We thus decided to initiate a comprehensive search for novel proteins capable of regulating TRs activity using an unbiased screening system. For this purpose, a series of pull-down assays was performed. The interactions between the recombinant protein fused to glutathione-S-transferase (GST-TRα or GST-TRβ) and around 50 epigenetic modification enzymes were tested. Many factors such as histone lysine methyltransferase SUV39h1, histone deacetylase HDAC1, previously shown to be co-regulators of other nuclear receptors were identified. We didn't obtain a coregulatory protein which shows a clear preference for one specific TR isotype. However, this unbiased screening enables us to identify TET3 as a novel interaction partner for TR.

TET3 belongs to TET family proteins, which were first identified as DNA hydroxylases. TET proteins catalyze iterative oxidation of methylated cytosine (5mC), thus acting as initiators of DNA demethylation process (refer to TET proteins part). Functional study of TET proteins revealed their dual role in transcriptional regulation, dependent or independent on its hydroxylase activity. TET proteins were shown to contribute to gene activation by removing the repressive DNA methylation marker or by maintaining the hypomethylated state at the active promoter or enhancer region. In addition, TET proteins could also serve as a scaffolding proteins recruiting other protein complex, such as Sin3A/NuRD complex and OGT, which shape the chromatin landscape by favoring a closed/open chromatin state and thus mediate gene repression/activation (refer to TET proteins part). Since we identified a physical interaction between TET3 and TR, my project aimed to explore whether and how such a versatile protein is involved in regulating TR activity.

Moreover, our study revealed that the interaction with TET3 is not specific to TR, since an interaction between TET3 and other members of the NR family was observed, such as AR (androgen receptor), ERR (Estrogen-related receptor) and RAR (retinoic acid receptor), suggesting that TET3 might be a general cofactor for nuclear receptors. The fact that there is no available antibody of sufficient quality to detect endogenous TET3 or TR proteins due to the low abundance of the two proteins in somatic cells, makes the *in vivo* functional study of TET3/TR interaction difficult. This prompted us to switch to another nuclear receptor, RAR, to study the functional consequence of TET3/RAR interaction. RAR is abundant in ES cells, and TET3 expression is induced during the differentiation process of ES cells (Dawlaty et al., 2013). In addition, RAR is the main mediator of retinoic acid (RA) during RA induced differentiation of ES cells (Mahony S et al, 2011). Combined deficiency of all three TETs has been reported to deplete 5hmC

and impaired ES differentiation (Jaenisch R et al, 2014). Thus both TETs and RAR are present and have important role during ES cell differentiation and embryonic development, making ES cells a good cell model to study the functional relevance of TET3/RAR interaction.

Project 1 (Revision in PNAS)

Title

Methylcytosine dioxygenase TET3 interacts with thyroid hormone nuclear receptors and stabilizes their association to chromatin

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Footnotes

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Abstract

Thyroid hormone receptors (TRs) are members of the nuclear hormone receptor family which act as ligand-dependent transcription factors. Here we identified TET3 as a new TR interacting protein increasing the cell sensitivity to T3. The interaction between TET3 and TR is independent of TET3 catalytic activity and allows the stabilization of TR and its preferential recruitment to the chromatin. This results in a potentiation of its transcriptional activity. So this study evidences a new mode of action for TET3 as a non-classical regulator of TR, modulating its stability and access to chromatin rather that its intrinsic transcriptional activity. This regulatory function might be more general towards the nuclear receptors since different members of the superfamily present the same interaction with TET3. Interestingly the differential ability of different TRα1 mutants to interact with TET3 might explain their differential dominant activity in patients carrying germline mutations.

Significance Statement

Thyroid hormone (T3) controls both developmental and physiological processes. Its nuclear receptors (TR) are transcription factors. Their expression is wide but their activity differs depending on the tissue. Availability of T3, cofactors involved in TR chromatin binding or activity but also TR itself modulates T3 response. Methyl dioxygenase TET3 is characterized here as a new TR partner. It stabilizes and promotes chromatin recruitment of TR increasing the sensitivity of the cell to T3. Mutations in TRα cause the RTHα symptom which severity varies with the particular mutation. Only some mutated TR can be stabilized by TET3. The availability of TET3 is a novel parameter modulating TR activity and its differential interaction with mutated TR might explain different severity of RTHα.

Introduction

Thyroid hormone (T3) is the main natural iodinated compound possessing a biological activity. It exerts a pleiotropic action on development and homeostasis, acting on most if not all cell types[1] . T3 acts directly on gene transcription by binding to the thyroid hormone receptors (TRs): TRα1, TRβ1 and TRβ2. They are respectively encoded by the *THRA* and *THRB* genes. In humans, mutations of either *THRA* or *THRB* cause the resistance to thyroid hormone syndrome (RTH). The severity of the disease is determined by the precise location of the mutation, dictating the ability of the mutated TR to respond to T3[2].

TRs as the other members of the nuclear receptor superfamily are ligand-regulated transcription factors consisting of three functional domains: the amino-terminal A/B domain, the DNA-binding domain (DBD) and the ligand-binding domain (LBD). TRs can bind to DNA on TR response Element (TRE) in absence of T3 and on most genes repress transcription until T3 binds and leads to activation[3]. Helix12 is the major structural element associated with this process. T3 triggers a dramatic shift of its position, leading to dissociation of corepressors and recruitment of coactivators, including coactivators that have the ability to change the chromatin micro-environment[4-6] . T3-binding also induces a rapid proteasome-mediated degradation of TRs which is associated with T3-dependent transcriptional activity[7]. TR availability and chromatin access is thus possibly an important level of modulation of T3 cellular response.

The goal of the present study was to identify new epigenetic regulators that interact with and modulate TR transcriptional activity using *in vitro* pull-down screening. This approach allowed us to identify TET3, a member of the ten-eleven translocation (TET) family proteins, as a novel partner for TRs. The TET proteins have been extensively studied as dioxygenase enzymes responsible for demethylation of methylated CpG dinucleotides by catalyzing the hydroxylation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC)[8,9]. Here we demonstrate a direct interaction between TET3 and TRα1, involving the CXXC and catalytic domains of TET3 and the helix12 of TRα1. This interaction stabilizes TRα1 by inhibiting ubiquitination and enhances TRα1 chromatin recruitment and transcriptional activity independent of its dioxygenase activity. Thus we discovered a novel way for TET3 to regulate transcription by modulating the protein turn-over and chromatin association of a transcription factor, here TRα1.

Results

TET proteins interact with TR

To find out new epigenetic modifiers involved in modulating TR activity, a series of pull-down assays was performed. The interactions between the recombinant protein fused to glutathione-S-transferase (GST-TRα or GST-TRβ) and around 50 epigenetic modification enzymes were tested. The NCoR corepressor and SRC3 coactivator were found to interact with both GST-TRα and GST-TRβ validating the screen. Many other factors such as histone lysine methyltransferase SUV39h1, histone deacetylase HDAC1, previously shown to be co-regulators of other nuclear receptors were also identified. TET3 was a novel interactor which came out (Fig.1A). Co-immunoprecipitation assays were performed for all three TETs to test whether the interaction with TRs can take place in HEK293T cells. As TETs are large proteins difficult to produce, only their catalytic domains were used as a first intention. The catalytic domain of TET3 strongly interacted with TRα1 (Figure 1B). This interaction was reduced by T3 addition in HEK293T cells but not in the pull down assay. The interaction with full-length TET3 was also validated by co-immunoprecipitation. As for the catalytic domain, a relative stronger interaction was observed in the absence of T3 (Fig.1C). This suggests that TET3 can interact with both the apo- and holo- conformations of TR, but that in a cell environment and in the presence of T3, this interaction is somehow balanced and displaced by the present “classical” co-activators. As the interaction with the catalytic domain of TET1 and TET2 was considerably weaker (Fig.1B), the rest of the study was limited to the interaction between TET3 and TRα1. We studied in parallel the interaction with TRβ1 and obtained the same results presented in figure S1.

TET3 and TRα1 interacts via the CXXC & catalytic domains in TET3 and AF2 domain in TRα1

A series of vectors was generated to express tagged and truncated TET3 (Flag) or TRα1 (Flag) proteins and to more precisely map the interacting domains (Fig.2A). Co-immunoprecipitations showed that both the CXXC domain and the catalytic domain of TET3 interact independently with TRα1 (Fig.2B). Conversely, the presence of the C-terminal helix12, commonly called AF2, of the ligand binding domain of TRα1 was found to be necessary for interaction with TET3 (Fig.2C).

TET3 modulates T3 response by regulating TRα1 protein levels

After identifying the interaction between TET3 and TRα1, we evaluated whether TET3 affected TRα1 activity. We first examined the effect of TET3 expression on TRα1 transcription capacity in a transient expression assay performed in HEK293T cells. Full-length TET3 enhanced TR transcriptional activity in a dose-dependent manner, while a TET3N mutant that cannot interact with TR, failed to do so (Fig.3A). The catalytic activity was not required for this effect to be observed, as demonstrated by using the $TET3^{H1077Y/D1079A}$ mutant (TET3mut) (Fig.3A) that lacks the dioxygenase activity (Fig. S2A) but retains the ability to interact with TET3 (Fig.S2B). Then we moved to cellular systems to look at the regulation of endogenous target genes. We used a cell line where TRα1 and TET3 proteins are not overexpressed and in which TRα1 target genes have been fully identified. These neural cells, called C17.2GSα cells[10] express in a stable manner a murine TRα1 with a double N-terminal tag (protein G fragment followed by streptavidin binding peptide). These cells also express endogenous TET3 at higher level than TET1 and TET2 (Fig.S3A). SiRNA-mediated knockdown of TET3 in C17.2GSα only partially silenced (60%) TET3 (Fig.S3A). This treatment did not alter the cell response to T3, as judged by RT-qPCR measurement of mRNA for *Epas1* and *Klf9*, 2 genes which expression level is strongly increased by the hormonal treatment (Fig.S3B). Given the limited efficacy of TET3 knockdown we used the CRISPR/Cas9 technology to knockout both copies of the *TET3* gene, in equivalent cells, called C17.2Sα, in which the protein G tag was omitted to prevent cross-reactivity of the tagged TRα1 with any IgG. A cell clone was identified (C17.2SαKO) with frameshift mutations on both alleles of the *TET3* gene (Fig.S4A). The absence of TET3 expression in this clone was confirmed by RT-QPCR (Fig.S4B). A cell clone without TET3 mutation and with a comparable level of TR (Fig.S4B) expression served as a control (C17.2SαC) in the following experiments. TET3 KO led to a decreased induction by T3 of *Epas1* and to a lesser extent, of *Klf9* expression (Fig.3B). Moreover, TET3 KO severely compromised level of SBP tagged TRα1 protein (Fig.3C) even though more SBPTRα1 transcript was detected in C17.2SαKO than in C17.2SαC cells (Fig.S4B). Interestingly, the same phenomenon was observed in C17.2GSα: the knockdown of TET3 also decreased TRα1 protein level (Fig.S3C), while the TRα1 mRNA level was not affected (Fig.S3A). Most importantly, the destabilization of TRα1 in C17.2-SαKO cells is the direct consequence of TET3 KO since TRα1 expression and the induction of its targets by T3 was rescued after re-introduction of TET3 by lentivirus infection (Fig 3B and 3C). The rescue is efficient but partial, correlated with the percentage of infected cells (Fig S4C). All these results suggested that TET3 is involved in modulating TRα1 protein level and that by doing so it also impacts on the regulation of T3 responsive genes.

TET3 stabilizes TRα1 by inhibiting its ubiquitination.

To further evaluate the ability of TET3 to regulate TRα1 protein level, we then examined the effect of TET3 on TRα1 protein stability in transfected HEK293T cells by adding cycloheximide (CHX), an inhibitor of protein translation. As expected, the protein level of TRα1 and TRα1ΔH12, that lacks helix12, quickly decreased over time. Addition of TET3 prevented the degradation of TRα1, but not of TRα1ΔH12, with which TET3 cannot interact (Fig.4A). The TET3mut that lacks dioxygenase activity (Fig.S2A) retained its interaction with TRα1 (Fig.S2B) and the capacity to stabilize TRα1 protein (Fig.S2C) by inhibiting its ubiquitination (Fig.S2D). These results show that TET3 regulates TRα1 protein stability and that this stabilization requires the direct interaction between the two proteins, but not the enzymatic activity of TET3.

As TRα1 is degraded *via* the ubiquitin-mediated proteasome, we then examined whether the over-expression of TET3 could modify the ubiquitination pattern of TRα1. Degradation was prevented by a cocktail of MG132 and E64D inhibiting respectively the proteasome *per se* and the lysosome mediated degradation of ubiquitinated proteins that might occur when proteasome is blocked. As expected this resulted in an accumulation of poly-ubiquitinated TRα1 in transfected HEK293T cells (Fig.4B). TET3 limited the amount of poly-ubiquitinated TRα1. Furthermore a similar blockade of degradation could partially rescue TRα1 expression in C17.2-SαKO cells (Fig.4C). Altogether these results suggest that TET3 protects TRα1 from degradation by limiting its poly-ubiquitination but also that in C17.2SαKO cells additional pathway(s) to proteasome and lysosome degradation are involved in clearing ubiquitinated proteins. Indeed MG132 and E64D treatment in those cells was not sufficient to fully restore TRα1 expression. Similar results were obtained in C17.2GSα cells (Fig.S3C).
Considering that TET3 protects TRα1 from degradation in an interaction-dependent manner, we hypothesized that the attenuated TET3/TRα1 interaction in the presence of T3, demonstrated by co-immunoprecipitation, should entail a more rapid degradation of TRα1. This hypothesis implies that TET3-mediated stabilization of TRα1 should be less active in the presence of T3. In agreement with published results[7] we observed that T3 accelerates the degradation of TRα1 in transfected HEK293T cells. In this system however TET3 extended the half-life of TRα1 both in the absence and presence of T3 (Fig.4E) implying that TET3 stabilizes TR even in the presence of T3.

Collectively, the convergence of results obtained in three independent cellular models strongly indicates that TET3 influences the cell sensitivity to T3 by preventing the ubiquitination and degradation of TRα1.

TET3 facilitates the recruitment of TRα1 to chromatin

We evaluated the possibility that TET3 may influence TRα1 cellular localization and/or chromatin association in addition to its effect on protein stability. Cell fractionation of transfected HEK293T cells confirmed that TRα1 is mainly recovered in the nucleus, but only a sub-fraction is chromatin associated. Importantly, co-expression of TET3 substantially increased the chromatin fraction of TRα1 (Fig.5A). This effect is also likely to require direct interaction, as TET3 has no visible effect on the chromatin bond fraction of TRα1ΔH12 (Fig.5A) and as TET3N, a truncated form of TET3 which does not interact with TRα1, could not promote TRα1 recruitment (Fig.5B). The enzymatic activity of TET3 is also dispensable to enhance TRα1 binding to chromatin (Fig.5B).

The potential role of TET3 in modulating the dominant negative effect of TRα1 mutants

Another situation where TET3/TRα1 interaction may have significant consequences is in patients with RTHα. The missense or frameshift mutations found in these patients confer dominant-negative properties to the mutant receptor. In each patient a mutated and a normal (WT) version of the receptor coexist and the activity of the mutated receptor prevents the normal activity of the WT version. This can be evidenced in transient expression assays where the co-expression of mutant and WT TRα1 results in impaired transactivation capacity, mimicking the situation found in cells of heterozygous patients[11] .The dominant negative effect varies with the ratio between mutant and WT receptors, and the type of mutation. There is a clear correlation between this *in vitro* property of the mutant receptor and the severity of the clinical phenotype. The mechanisms responsible for the dominant negative action are

unclear, and probably combine protein stability, balance between corepressor and coactivator interactions, competition between intact and mutant receptors, and half-life of histone marks. We tested the possibility that the capacity of the mutant receptor to interact with TET3 could determine the stoichiometry between mutant and WT receptors, and thus influence the dominant negative activity of the mutant receptor, and the disease severity.

We used here a panel of natural and artificial mutations altering helix12, and assess both the influence of the mutation on TET3 interaction and dominant-negative property. TRα1^{E403X} [12] and TRα1^{N359Y} [13] have been found in two patients, TRα1^{E403X} patient having a more severe resistance to T3 treatment. TRα1^{L400R} is lethal in a mouse knock-in model[14](Fig6A). Among these mutant receptors, only TRα1^{N359Y} and TRα1^{L400R} that retain the helix structure of helix12, interacted with TET3-Cat (Fig.6B) as expected from our previous conclusion that helix12 is required for TET3/TRα1 interaction. As expected TET3 prevented the degradation of TRα1^{L400R} but not TRα1^{E403X} in the transfected HEK293T cells treated with CHX (Fig.6C). Accordingly the recruitment of TRα1^{L400R} but not TRα1^{E403X} to the chromatin was enhanced in the presence of TET3 (Fig.S5). We also performed transient co-expression of TRα1/TRα1^{L400R} or TRα1/TRα1^{E403X} to measure the dominant-negative activity of these two mutant receptors, which was similar. However in the presence of TET3, TRα1^{E403X} dominant-negative activity is less potent whereas TRα1^{L400R} dominant activity is not affected (Fig.6D).
A simple explanation would be that TET3 stabilizes TRα1 and TRα1^{L400R} but not TRα1^{E403X}, and thus influence the stoichiometry and the capacity of the cells to respond to T3 as illustrated on the scheme (Fig.6E).

Discussion

In this study, we demonstrate that TET proteins can interact with the nuclear receptors for thyroid hormone, TRα1 and TRβ1. Focusing on the most salient TET3/TRα1 interaction, we found that the presence of TET3 has three consequences: a) it increases the half-life of TRα1 by reducing ubiquitination and degradation b) it facilitates its recruitment to chromatin c) it increases its capacity to mediate transcriptional activation upon ligand binding. These three effects do not rely on the catalytic activity of TET3, and require the presence of the C-terminal helix12 of TRα1. There are thus most probably mutually dependent. Although other possibilities exist, a simple hypothesis would be that, by tethering TRα1 to the chromatin, TET3 protects it from ubiquitination and proteasome degradation, and favors the activation of gene expression in the presence of T3. In many cell-types, the presence of TET3 would thus increase the cellular sensitivity to T3 stimulation. This role of TET3 is not limited to TRα1, since similar results have been obtained for TRβ1. It might even be more general, and regulate the hormone sensitivity of the cell to a host of different nuclear receptors since the AF2 domain involved in the interaction is well conserved in this family of transcription factors and that the interaction has been observed with AR and ERR (Fig.S6).
We previously described that all T3 target genes are not equally sensitive to the amount of TR expressed. Endogenous TR are hardly expressed in the C17.2 cell line, however a limited number of genes including *klf9* are nonetheless regulated by T3 in these cells. For those genes expressing significantly higher level of TR did not further induce the regulation. In contrast most of the genes, including *epas1* revealed their

regulation only in these conditions. We observed in the present work that the induction of *epas1* gene was more potentiated by TET3 than *klf9* expression. The stabilization of TRα1 in these conditions might account for this differential effect. In patients with TRα1 mutations, the presence of TETs should also finely tune the negative influence of the mutant receptor.

The fact that T3 tends to destabilize the interaction between TET3 and TRα1, as judged by co-immunoprecipitation, is counterintuitive, as this seems to imply that TET3 should have no influence in T3 treated cells. It should be noticed however that *in vitro* pull-down assays do not indicate any influence of T3 on the interaction capacity of TRα1 when isolated from the cellular context. It is therefore likely that the reduced interaction observed in T3 treated cells is a consequence of competition with other proteins able to interact with TRα1 in a T3 dependent manner. As histone acetyl transferases and TET3 share the same interaction domain on TRα1 ligand binding domain, T3 addition probably provokes a competition between coactivators and TET3. We thus believe that this competition only occurs after the tethering of TRα1 to the chromatin by TET3. This conclusion is in agreement with our observation that addition of T3 reduced the half-life of TRα1, as ligand binding restricts the stabilization effect of the TET3/TRα1 interaction. Thus in the cellular context in presence of TET3, TRα1 is more stable, recruited to the chromatin, leading to an increase induction of TR target genes. T3 binding would compete TET3 out, allowing the transcription and then the recycling of the receptor via its degradation.

In addition TET3/TRα1 interaction could influence the DNA methylation status near TRα1 binding sites in some cell types. Indeed as anticipated from their hydroxymethylase activity TETs can modulate transcription by adjusting levels of DNA methylation at promoters. Accordingly, both TET1 and 5hmC often localize to transcriptional start sites (TSSs)[15,16]. However such phenomenon was not observed in the cell systems that we used. Moreover all the observations gathered in the present study argue for an action of TET3 that does not rely on its catalytic activity. So at this point we have no evidence that the interaction modulates TET3 activity or the transcriptional activity of TRα1 *per se*. This is not the first time that an enzymatic independent activity of TET has been demonstrated. Accumulating evidences indicate that TET proteins can regulate transcription *via* different pathways that do not all involve their enzymatic activity. In several instances, TETs regulate transcription by serving as scaffolding proteins that help to bridge transcriptional factors and cofactors. For example, TET1 was shown to interact with mSin3A[15] and the MBD3/NuRD complex[17] which associate with HDAC1 and HDAC2 histone deacetylases to maintain histones in a deacetylated form, thereby repressing transcription. TET1 genomic location is also reported to overlap with the one of the Polycomb repressive complex PRC2. As a direct interaction between TET1 and PRC2 proteins could not be demonstrated, it seems that TET1 facilitates PRC2 chromatin binding by decreasing DNA methylation levels at PRC2 target genes[18,19]. Finally all three TETs were reported to interact with the O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT), to allow its recruitment to chromatin[20,21]. The ensuing glycosylation of histone H2B and the subsequent trimethylation of histone H3 at lysine 4(H3K4me3)[22,23] triggers gene transcription
Our study is the second one to establish an interesting direct link between two families of proteins, nuclear receptors and TET methylcytosine dioxygenases, which both received considerable interest for different reasons. It was observed previously that peroxisome proliferator-activated receptor-γ (PPARγ)

has the ability to direct local demethylation around its binding sites and to co-immunoprecipitate with TET1[24]. The transactivation capacity of the glucocorticoid receptor (GR) in neural stem cells was also found to be TET dependent but the underlying mechanism was not clarified[25] and no evidence provided for an interaction between GR and TET. Our study differs from the previous mainly by concluding that the catalytic activity of TET3 was not necessary to enhance TRα1 function. In our original screen TET1 and TET2 were also interacting with TRα1, even if the interactions were weaker. Given the high homology with TET3, they might also modulate T3 cell sensitivity but proper experiments are needed to ascertain this hypothesis.

The original mechanism involved here, *ie* stabilization and enhanced chromatin recruitment is very different from the one classically described for nuclear receptor co-activators. However TET3 can still qualify as a bona fide co-activator of TR that increases their transcriptional activity. Given the *in vitro* interaction that we observed between TETs and several other nuclear receptors we believe that the modulation of hormonal regulation by TET might be a more general event.

Materials and Methods

Plasmids, Antibodies and Drugs

Flag-tagged TET1, TET2, TET3[26] and pCEMM-GS-TRα1/TRβ1[10] vectors were generated as previously described. TET3/TRα1 deletion mutants were generated by PCR amplification and cloned into p3xflag-cmv-9 (Sigma). The TET3 (H1077Y/D1079A) mutant, pCEMM-GS-TRα1^{L400R} and pCEMM-GS-TRα1^{E403X} were created by PCR-directed mutagenesis. Monoclonal anti-FLAG® M2-Peroxidase (HRP) (A8592,Sigma), monoclonal anti-β-Actin (A5316,Sigma), anti-GFP (ab290,Abcam), anti-β-tubulin (ab6046, Abcam), anti-TRα1/β1 (SC-739, SantaCruz), anti-Histone3 (ab1791, Abcam), anti-Myc (ab9106,Abcam) and anti-Ubiquitin (Z0458,Dako and VU101, Life sensor) were used for western blot or co-immunoprecipitation. Chemicals used in this study include: 3,3',5-Triiodo-L-thyronine sodium salt (T3, sigma), Cycloheximide (CHX, Calbiochem), MG132 (Calbiochem), E64D (Enzo Life Sciences). When indicated 5.10^{-8} M or 10^{-8} M of T3, 80µg/ml of CHX, 5.10^{-6} M of MG132 and 2.10^{-5} M of E64D was used.

Immunoprecipitation

Immunoprecipitations were carried out using HEK293T cells as previously described[26]. Magnetic M2 (Sigma), magnetic M280 (Dynabeads® M-280, Invitrogen) or Streptavidin beads (Agilent Technologies) were used when indicated.

TET3 KO by CRISPR/Cas9 and TET3 Rescue by Lentiviral vector in C17.2 Cell Line

The pCEMM-STRα1 vector encodes a SBP (streptavidin binding peptide) tagged murine TRα1 and downstream IRES-GFP cassette that ensures co-expression of GFP from the same CMV transcription promoter. pX459-TET3 guide RNA vector contains a single guide RNA against TET3, a cas9 protein coding sequence and a puromycin resistance gene. The targeted sequence of the guide RNA was designed as described before and no off-target was identified[27,28]. The two constructs were transfected into C17.2 cells. Puromycin resistant cells (1µg/mL) were FACS sorted to select cells expressing both GFP and SBP-TRα1. Sorted cells were cloned and amplified, genotypes were determined by sequencing. One clone

with frameshift mutations for TET3 on both alleles was chosen (C17.2SαKO) for further analyses. A clone with comparable TR level (C17.2Sα), issued from a transfection with a PX459 empty vector and pCEMM-STRα1 was used as a control (C17.2SαC). Lentiviral particles for hTET3 over expression (amsbio; LVP876) were used to rescue TET3 expression in C17.2SαKO cells. A blasticidin-RFP fusion dual marker was expressed under the same promoter. Blasticidin resistant (1,5ug/mL) infected cells (C17.2Sα-R) were used for further experiments.

RNA Extraction and qPCR Measurements

Total RNA was extracted with the Macherey-Nagel RNA II kit according to the manufacturer's recommendations and quantified with Nanodrop (Thermo Scientific). 1µg of RNA was reverse transcribed using M-MLV reverse transcriptase (Promega). Relative Quantitative PCRs were performed in 96-well plates, using the SYBRGreen mix (BioRad iQ supermix) on the CFX96 real time system machine (BioRad). Expression levels were calculated using the $2^{-\Delta\Delta(Ct)}$ method and 36B4 as the normalizer. The sequences of the primers used are provided in supplemental table1. Error bars were shown as mean ± SD.

Cell Fractionation

Nuclear–cytoplasmic fractionation was done using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Cell Culture and Transient Expression Assays

C17.2 cells were maintained in DMEM (Invitrogen) supplemented with 12% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) antibiotics. Human HEK293T and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. These cells were grown at 37 °C in 5% CO2. *TransIT*®-LT1 Transfection Reagent (Mirus) was used for transfection according to the manufacturer's instructions. Luciferase assay was carried out using HEK293T cells as previously described[29]. Luciferase activity was normalized against the β-gal activity and expressed as arbitrary units. All assays were performed in at least triplicates and the data are presented as means +/- standard deviation.

Aknowledgements

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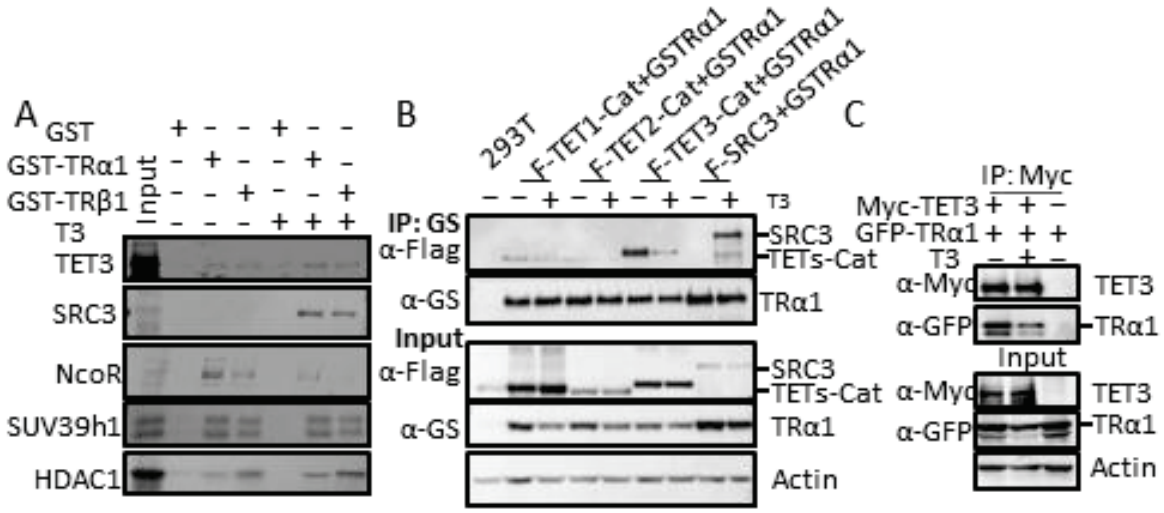
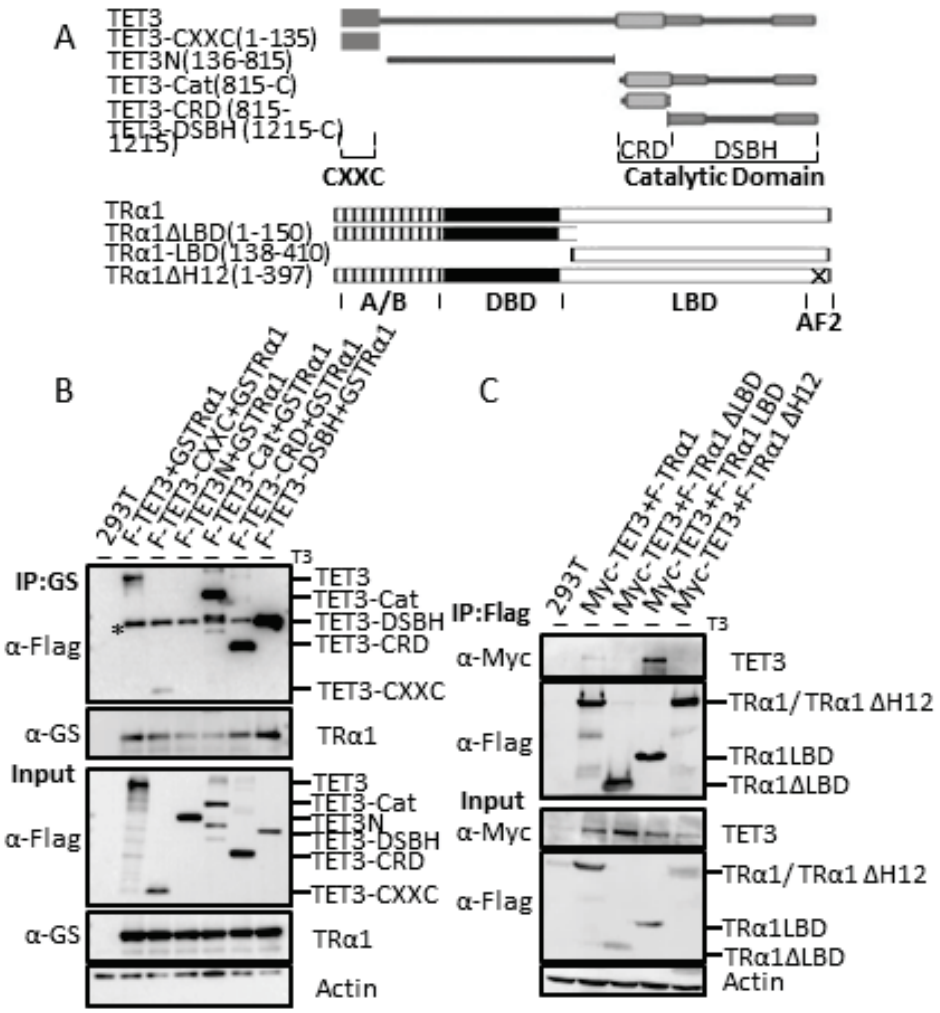
Figures and Figure legends

Fig.1: Identification of a hormone modulated interaction between TET3 and TR



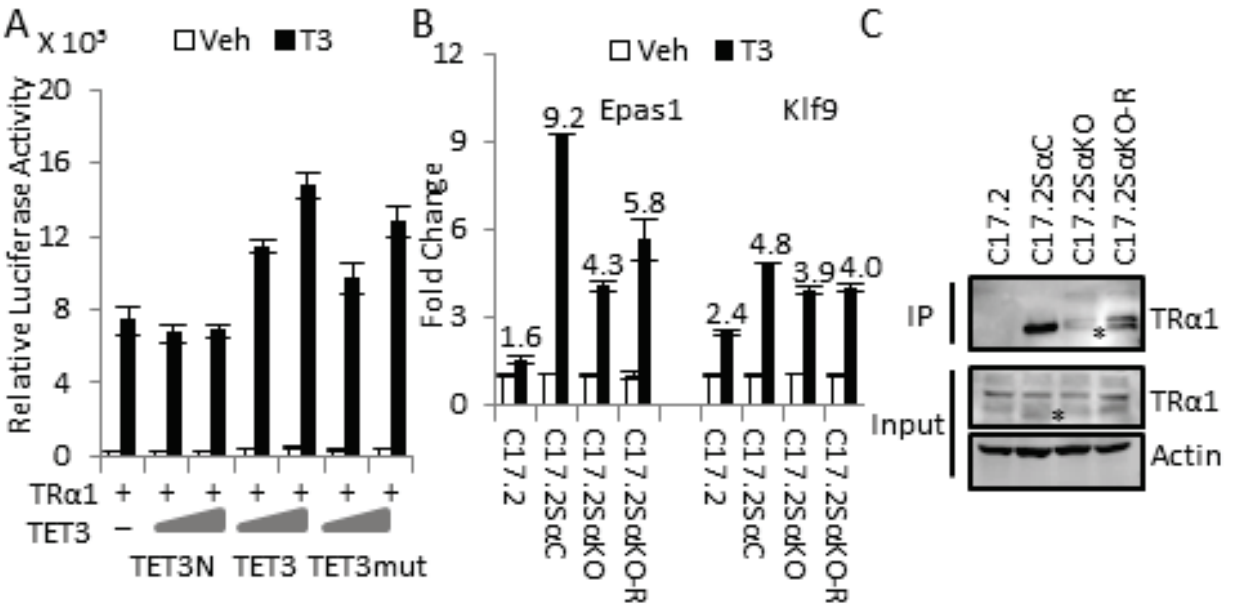
(A) Interaction between TET3 and TR identified by GST pull-down assay. GST pull-down assay was performed using recombinant GST-TRα1 or GST-TRβ1 proteins and lysates from HEK293T cells overexpressing TET3,SRC3,NcoR,SUV39h1 or HDAC1 in the presence or absence of T3 (5.10^{-7} M). (B) Interaction between GS-TRα1 and Flag-tagged catalytic domain of TETs (F-TETs-Cat) or SRC3 (F-SRC3) identified by co-immunoprecipitation assay. Whole-cell extracts from HEK293T cells co-transfected with indicated plasmids, treated or not with T3 (5.10^{-8} M) before collection, were used for Immunoprecipitation using M280 beads that retain GS tag. Co-precipitated TETs-Cat or SRC3 were detected by western blotting using an anti-Flag antibody. SRC3 was used as a positive control for all co-immunoprecipitations as TR/SRC3 interaction is well-known to be T3 dependent. Actin serves as a loading control. (C) Effect of T3 treatment on GFP-TRα1 interaction with Myc tagged full-length TET3 (Myc-TET3) identified by co-immunoprecipitation assay. Myc-TET3 and GFP-TRα1 were transfected in HEK293T cells and immunoprecipitated using an anti-Myc antibody, co-immunoprecipitated GFP-TRα1 was detected by western blotting using anti-GFP antibody.

Fig.2: The CXXC and catalytic domains of TET3 and the AF2 domain of TRα1 conferred the interaction between the two proteins



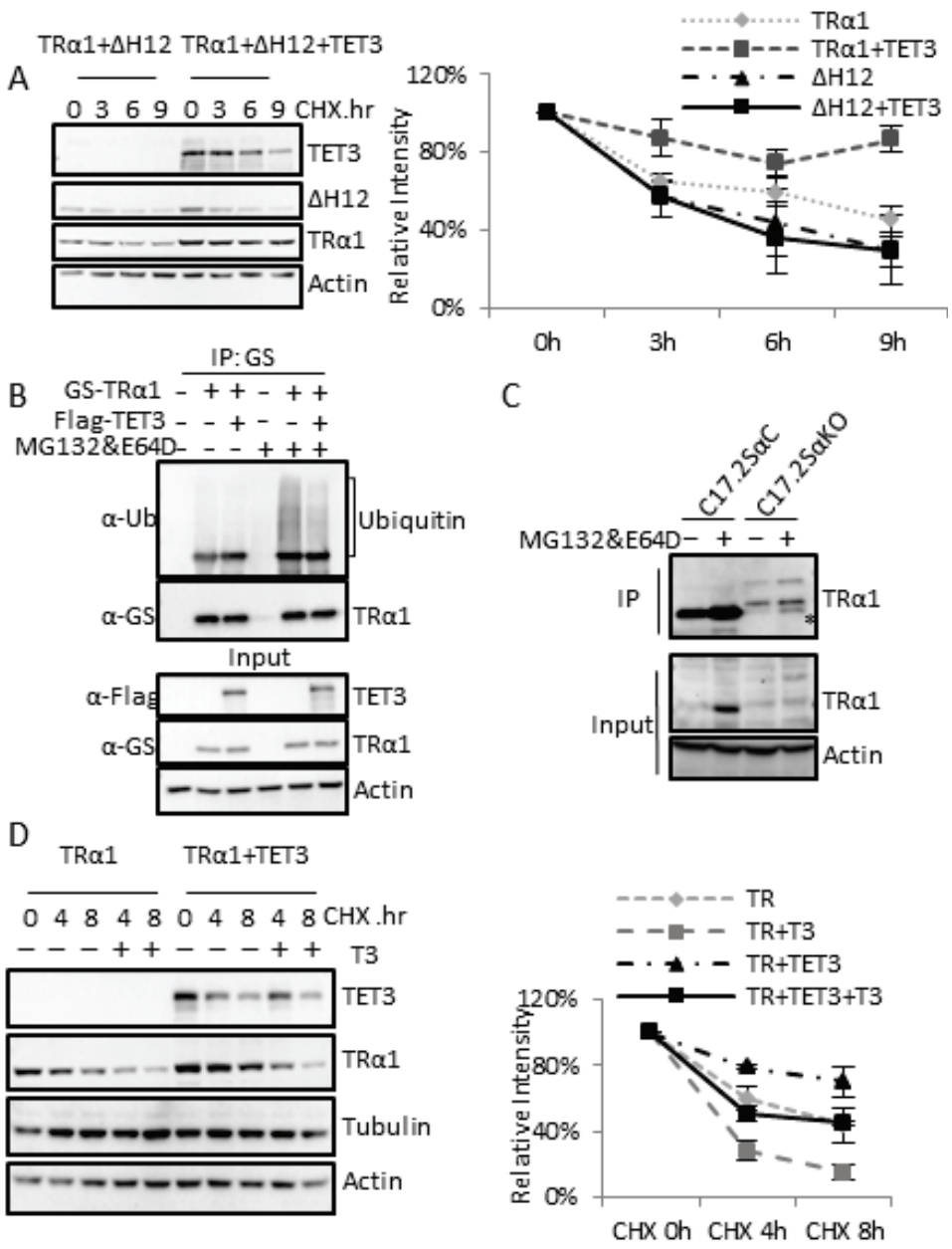
(A) Schematic representation of full-length and various truncation mutants of TET3 and TRα1. CXXC, CXXC domain; CRD, Cat, catalytic domain; cysteine-rich domain; DSBH, double-stranded beta-helix domain; DBD, DNA binding domain; LBD, ligand binding domain; AF2, activation function domain 2. (B and C) Co-immunoprecipitation assays identifying the interacting regions between TET3 and TRα1. Whole-cell extracts were prepared from HEK293T cells co-transfected with indicated plasmids. (B) GS-TRα1 was precipitated with M280 beads, co-precipitated F-TET3 mutants were detected using Flag antibody. Asterisk indicates band for TRα1 that is recognized thanks to the cross-reaction between anti-Flag antibody and its GS tag; (C) Flag beads were used to precipitate TRα1 and different truncation mutants, co-precipitated TET3 was detected with anti-Myc antibody.

Fig.3: TET3 regulates TRα1 activity by modulating TRα1 protein level



(A) Relative luciferase activity in HEK293T cells co-transfected with TRα1 and different TET3 mutants. HEK293T cells were transiently transfected with TRα1, DR4-luciferase and 2 doses of 3 TET3 constructs: without CXXC that lost interaction with TR (TET3N); wild-type TET3 (TET3) and enzymatic dead (TET3mut). Luciferase activities (upper panel) were measured 24 h after T3 (10^{-8}M) treatment, the triangles represent increasing amount of TET3 constructs. (B) TR target genes expression is regulated by TET3 levels in C17.2Sα cells. RNAs were extracted from indicated cells: control cells (C17.2SαC), TET3 KO cells (C17.2SαKO) and lentivirus infected C17.2SαKO cells (C17.2SαKO-R) treated or not with T3 (10^{-8}M). Relative expression level of TRα1 target genes (*Epas1* and *Klf9*) were examined by relative RT-QPCR. 36B4 is used as the normalizer and vehicle treated cells as the reference set to 1. The induction triggered by T3 was indicated on the top of the black bar. (C) Effect of TET3 expression levels on TRα1 protein level in C17.2Sα cells. Streptavidin beads were used to precipitate SBP tagged TRα1 from indicated cell lines as described in (B). Western blot for TRα1 was detected by TRα1 antibody, asterisk indicates band for TRα1.

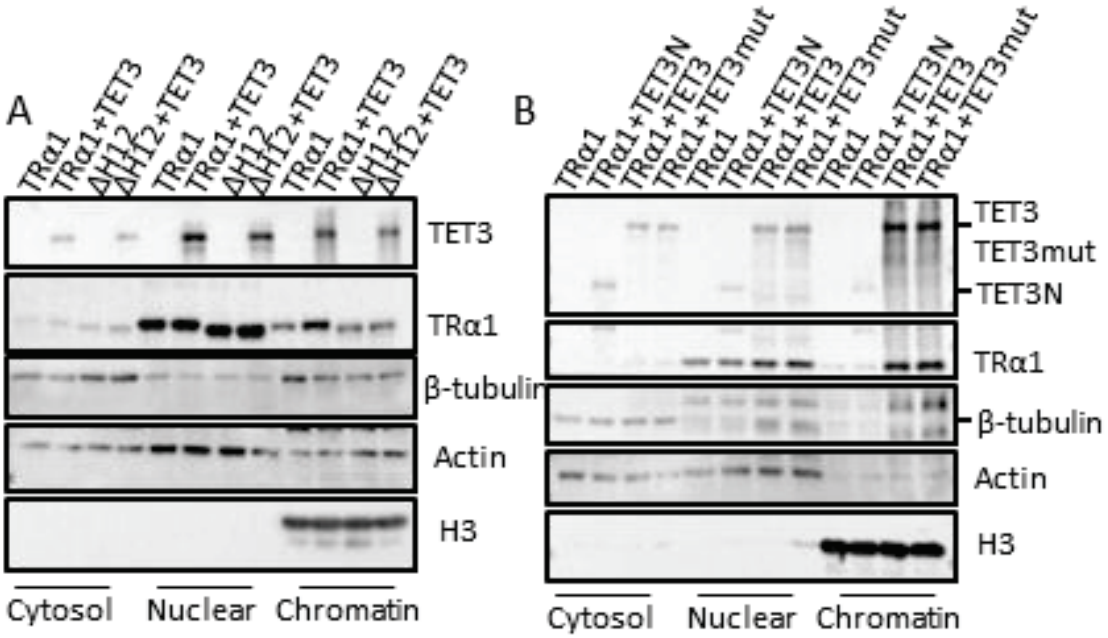
Fig.4: TET3 stabilizes TRα1 by inhibiting its ubiquitination



(A) Effect of TET3 on protein turnover of wild-type TRα1 (TRα1) and Helix 12 deletion mutant (ΔH12). HEK293T cells were co-transfected with Flag-TRα1 (TRα1) and GS-TRα1ΔH12 (ΔH12) with or without Flag-TET3 (TET3). Lysates were prepared from cells treated with cycloheximide (CHXfor indicated periods of time, protein levels of TRα1 or ΔH12 were detected by anti-Flag and anti-GS antibodies respectively (left panel). Band intensities of TR corrected by actin signals were plotted (right panel). The abundance at each time point was calculated relative to the abundance at time 0. (B) Ubiquitination pattern of TRα1 in the presence or absence of TET3. Whole-cell extracts from HEK293T cells transfected with indicated plasmids and treated or not with MG132&E64D before collection were

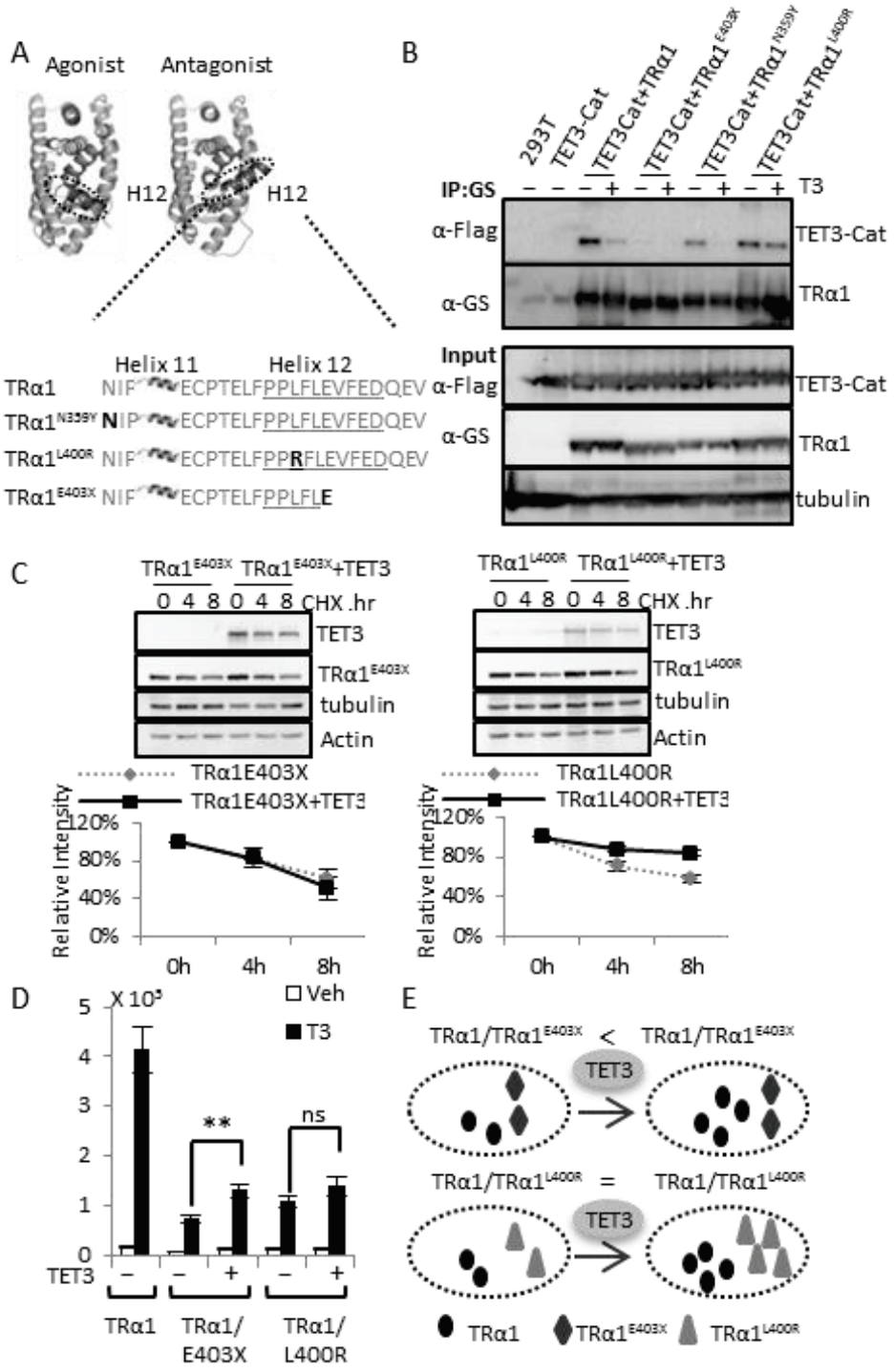
immunoprecipitated using M280 beads. Half of the immunoprecipitated TRα1 was subjected to western blotting as loading control the other half was used to determine the ubiquitination level of TRα1 with an anti-ubiquitin antibody. The smear indicates the poly-ubiquitination of TRα1. (C) Effect of MG132&E64D treatment on TRα1 protein level in C17.2Sα cells. Lysates prepared from control or TET3KO C17.2Sα cells pre-treated or not with MG132&E64D were incubated with streptavidin beads to precipitate SBP tagged TRα1. TRα1 was detected by TRα1 antibody, asterisk indicates band for TRα1. (D) Effect of TET3 over-expression on TRα1 protein turn-over in the absence or presence of T3. HEK293T co-transfected with GFP-TRα1 (TRα1) in the presence or not of Flag-TET3 (TET3) were treated with T3 (5.10^{-8}M) and/or CHX for indicated periods of time. Lysates were subjected to western blot (left panel), protein level of TET3 and TRα1 were respectively detected by anti-Flag and anti-GFP antibody. The intensity of TRα1 signals corrected by tubulin + actin signals were plotted on the right panel as 100% was set for the intensity measured for CHX 0h exposure.

Fig.5: Interaction between TET3 and TRα1 enhanced TRα1 recruitment to chromatin



(A) Effect of TET3 over-expression on the subcellular distribution of TRα1 or TRα1ΔH12. GS-TRα1 (TRα1) or GS-TRα1ΔH12 (ΔH12) were co-transfected with or without Flag-TET3 (TET3) in HEK293T cells. After cell fractionation cytosol, nucleus and chromatin fractions were subjected to western blotting. TET3 or TRα1 were respectively detected by anti-Flag and anti-GS antibody. β-tubulin, actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin. (B) Influence of TET3, TET3mut or TET3N over-expression on TRα1 subcellular distribution. Same fractionation experiment as in (A) was done for HEK293T cells co-transfected with GS-TRα1 (TRα1) and different Flag-tagged mutants of TET3.

Fig.6: Role of TET3 mediated stabilization of TRα1 on the dominant negative effect of TRα1 mutants



(A) Schematic of different TRα1 mutants. TRα1^{N359Y} has a mutation before helix 11 and an intact helix 12; TRα1^{L400R} has a point mutation in helix 12, and TRα1^{E403X} has a truncated helix 12. (B) Identification of

differential interactions between TET3 and different TRα1 mutants by co-immunoprecipitation. Flag-TET3Cat (TET3-Cat) and different GS-tagged mutants of TRα1 (TRα1) were transfected in HEK293T cells treated or not with T3 (5.10^{-8}M), TRα1 mutants were immunoprecipitated with M280 beads, co-precipitated TET3-Cat was detected with anti-Flag antibody. (C) Identification of differential stabilization of $TR\alpha1^{E403X}$ and $TR\alpha1^{L400R}$ by TET3. HEK293T cells co-transfected with $TR\alpha1^{E403X}$ or $TR\alpha1^{L400R}$ in the presence or absence of Flag-TET3 (TET3) were treated with CHX for 4h or 8h. Whole cell lysates were subjected to western blotting (upper panel). Protein level of TET3 or TRα1 mutants were detected by anti-Flag and anti-GS antibodies respectively. The intensity of TR mutant signals corrected by tubulin + actin signals were plotted on the lower panel as 100% was set for the intensity measured for CHX 0h exposure. (D) Modulation of the dominant negative effects of $TR\alpha1^{E403X}$ or $TR\alpha1^{L400R}$ by TET3 in luciferase assay. HEK293T cells were transiently transfected with DR4-luciferase, TRα1, TRα1/TR mutants(7/1) and TET3. Luciferase activities were measured 24 h after T3 (10^{-8}M) treatment. The asterisks indicate the significance of difference between the two black bars. (E) Working model for TET3 modulation of the dominant negative effects of TRα1 mutants. In the case of $TR\alpha1^{E403X}$ the ratio of TRα1/ $TR\alpha1^{E403X}$ is higher in the presence of TET3, so the cells are more responsive to T3 treatment, thereby the repressive effect of $TR\alpha1^{E403X}$ is less potent in the presence of TET3, while in the case of $TR\alpha1^{L400R}$ the ratio of TRα1/ $TR\alpha1^{L400R}$ doesn't change, so the cell response to T3 doesn't change either.

Supplementary Data

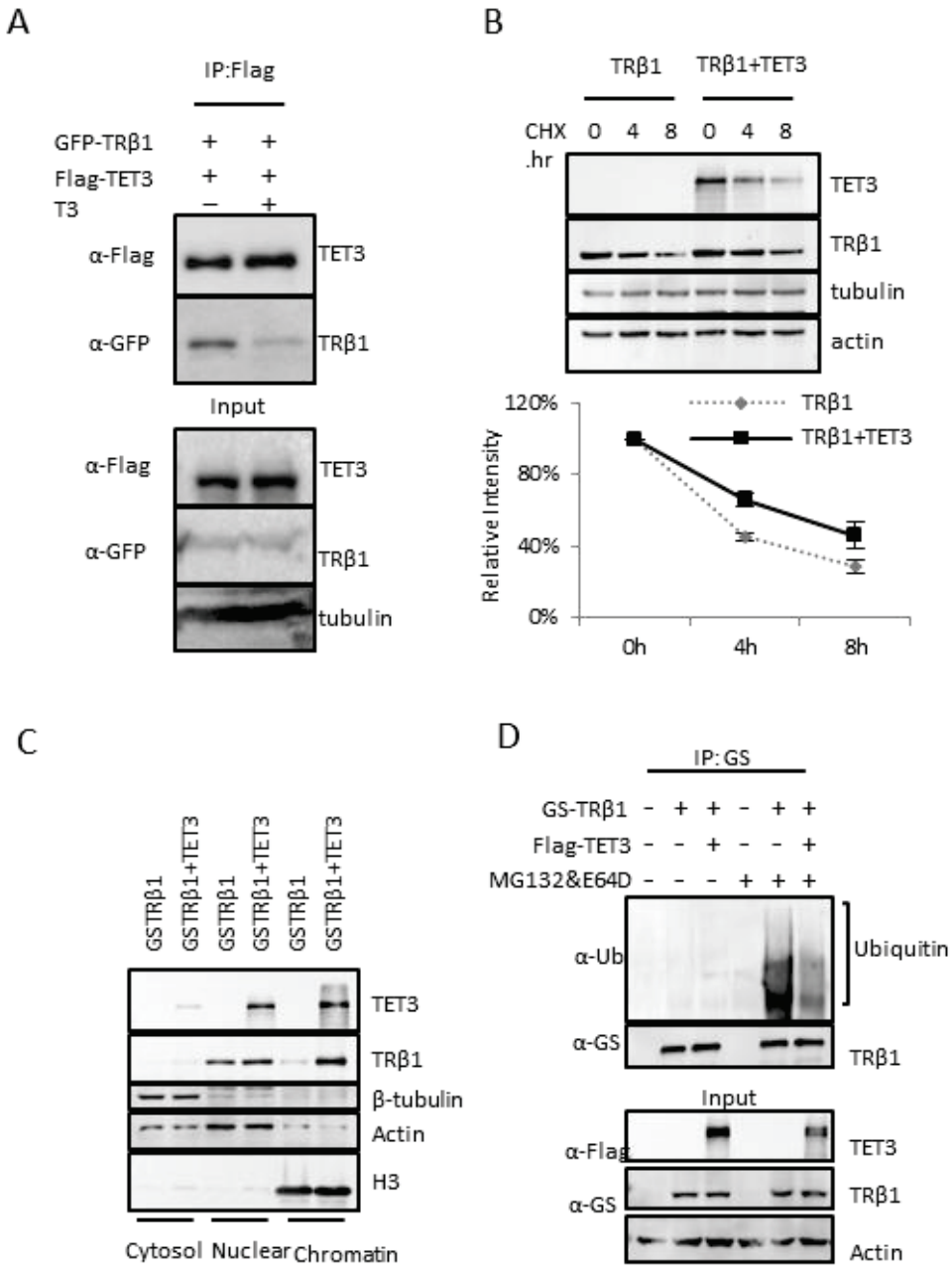
RNA interference in C17.2GSα cells

RNA interferences were performed in C17.2GSα cells that express in a stable manner a murine TRα1 with a double N-terminal tag (protein G fragment followed by streptavidin binding peptide). Small interference RNAs (siRNA) against TET3 were purchased from Dharmacon (siGenome Tet3 siRNA smart pool and non-targeting pool) and were transfected using lipo3000 (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence Staining

For immunofluorescence staining of 5hmC, HeLa cells were washed with PBS prior to fixation in 4% fresh paraformaldehyde in PBS for 15 min. Then cells were treated 30 min with at 37°C 2N HCl for, 20 min at room temperature with 100mM Tris-HCl (pH 8.0) for neutralization and blocked with 5% BSA in PBS for 1h at 37°C. 5hmC primary antibody was incubated overnight at 4°C and secondary antibody at room temperature for 1h. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI). For immunofluorescence staining in C17.2 cells, C17.2Sα or C17.2SαKO cells were washed with PBS prior to fixation in 4% fresh paraformaldehyde in PBS for 15 min. Fixed cells were treated with 1% triton-100 for 10min, followed by DAPI staining for 15min at room temperature. Images were acquired using Zeiss Inverted fluorescence microscope Axioobserver 7.

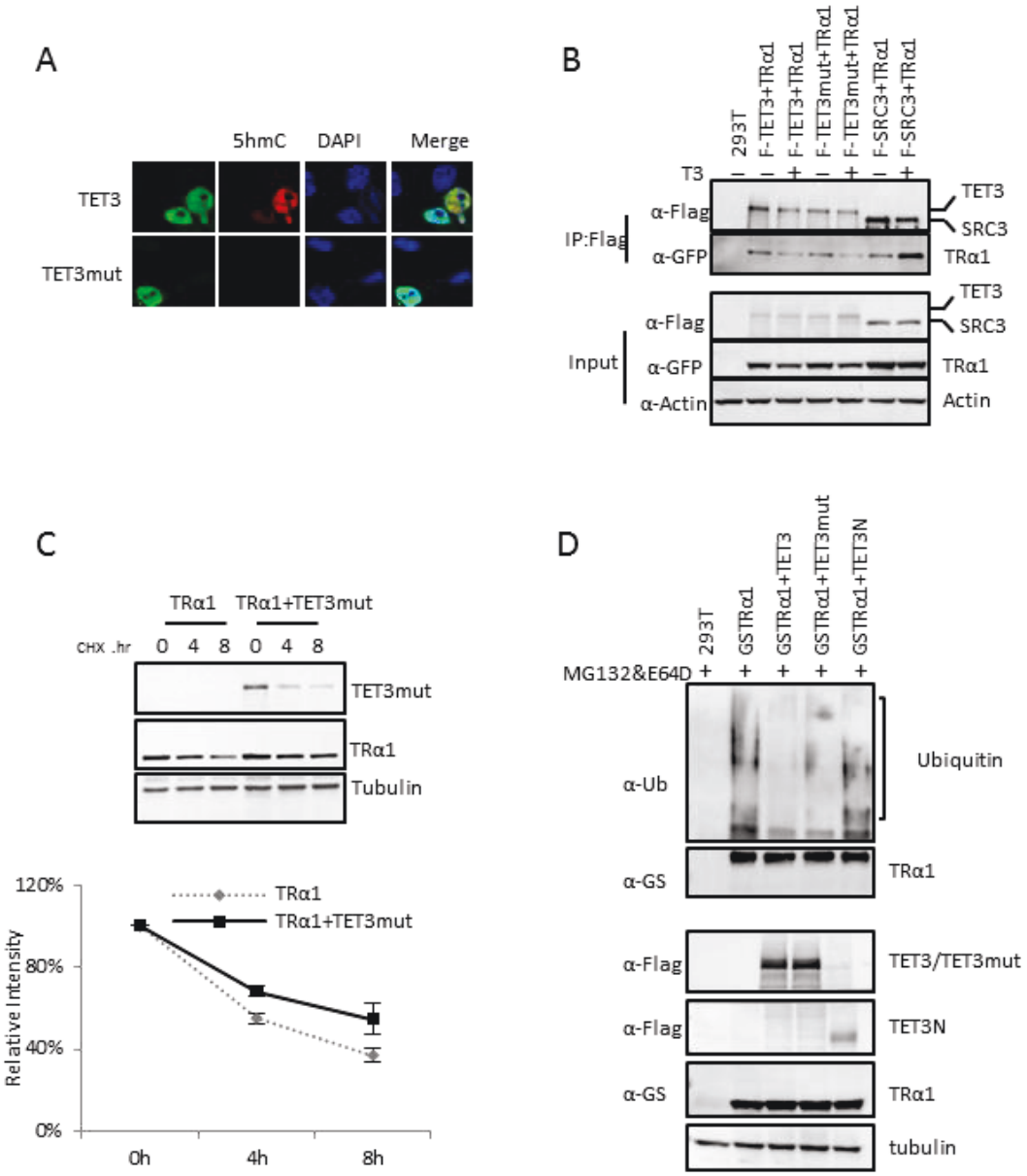
Fig.S1 TET3 interacts with, stabilizes and enhances the recruitment to chromatin of TRβ1



(A) Effect of T3 treatment on GFP-TRβ1 interaction with full-length Flag-TET3. Flag-TET3 and GFP-TRβ1 were transfected in HEK293T cells and immunoprecipitated using M2 beads, co-immunoprecipitated GFP-TRβ1 was detected by western blotting using anti-GFP antibody. (B) Effect of TET3 on protein turnover of TRβ1. HEK293T cells co-transfected with GS-TRβ1 (TRβ1) in the presence or absence of Flag-TET3 (TET3) were treated with CHX for 4h or 8h. Whole cell lysates were subjected to western blotting (upper panel). Protein levels of TET3 and TRβ1 were detected by anti-Flag and anti-GS antibodies respectively. The intensity of TRβ1 signals corrected by tubulin + actin signals were plotted on the lower panel as 100% was set for the intensity measured for CHX 0h exposure. (C) Effect of TET3 over-

expression on the subcellular distribution of TRβ1. GS-TRβ1 was co-transfected with or without Flag-TET3 (TET3) in HEK293T cells. Cells were fractionated using NE-PER nuclear and cytoplasmic extraction reagents kit. Cytosol, nucleus and chromatin fractions were subjected to western blotting. Protein levels of TET3 or TRβ1 were respectively detected by anti-Flag and anti-GS antibody. β-tubulin, actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin. (D) Ubiquitination pattern of TRβ1 in the presence or absence of TET3. Whole-cell extracts were prepared from HEK293T cells transfected with indicated plasmids. Cells were treated with or without MG132&E64D before collection. Immunoprecipitates were obtained using M280 beads, half of the immunoprecipitated TRβ1 was subjected to western blotting as loading control, while the other half was used to determine the ubiquitination level of TRβ1 by incubating with an anti-ubiquitin antibody. The smear indicates the poly-ubiquitination of TRβ1.

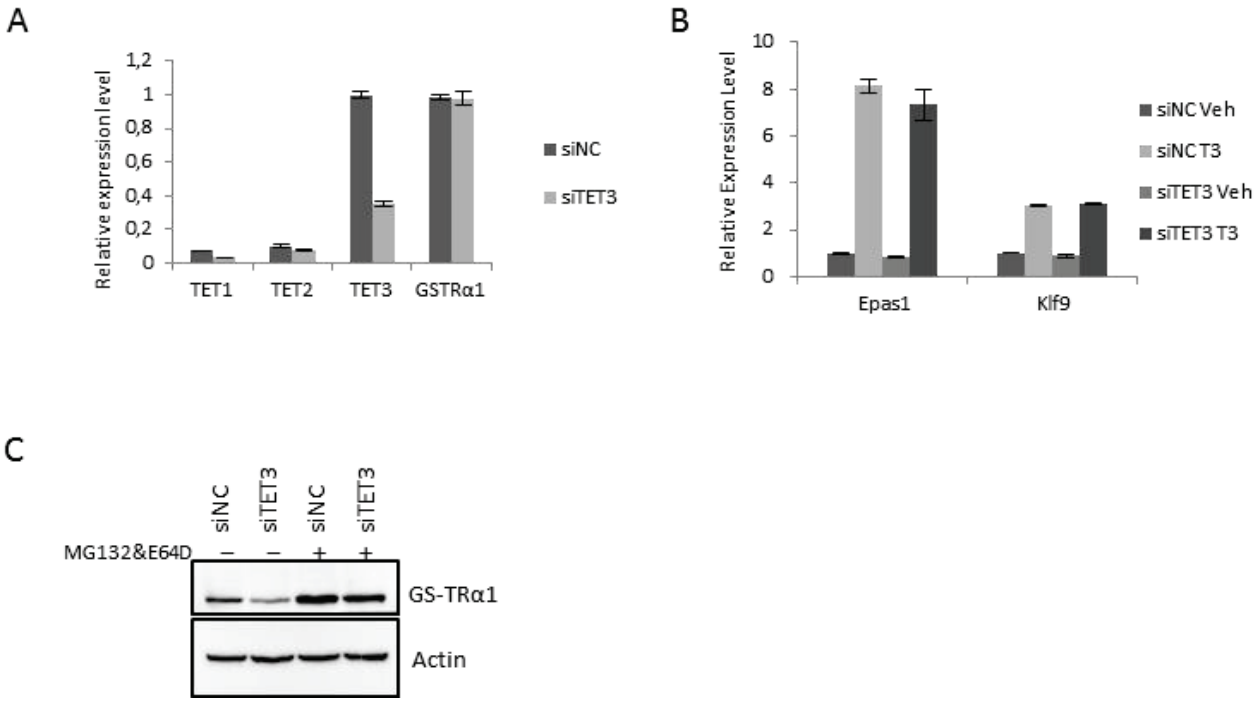
Fig.S2 Enzymatic dead mutant of TET3 retains the ability to interact with and to stabilize TRα1



(A) TET3mut lost its enzyme activity measured by immunofluorescence. 5hmC levels were analyzed in Hela cells transfected with Flag-TET3 (TET3) or Flag-TET3mut (TET3mut). Expression of TET3 constructs were revealed with the Flag antibody and presence of 5hmC with the 5hmC antibody. 5hmC staining was performed as described in supplementary *Materials and Methods*. (B) Both TET3 and TET3mut interact with TRα1 as identified by co-immunoprecipitation. Whole-cell extracts were prepared from HEK293T cells co-transfected with GFP-TRα1 (TRα1) and Flag-TET3 (F-TET3) or Flag-TET3mut (F-TET3mut) or Flag-SRC3 (F-SRC3) treated or not by T3 (5.10-8 M) before collection. Immunoprecipitates were obtained using M2 magnetic beads. Co-immunoprecipitated GFP-TRα1 was detected by western blotting

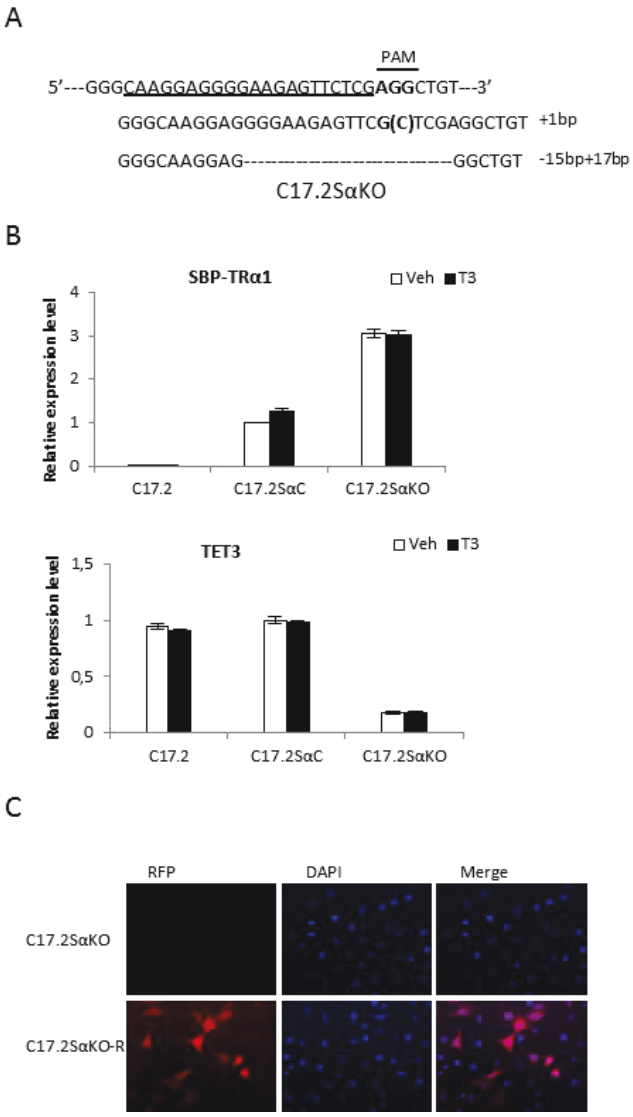
using anti-GFP antibody, actin was used as a loading control. (C) Effect of TET3mut on TRα1 protein turnover. HEK293T cells co-transfected with GS-TRα1 (TRα1) in the presence or absence of Flag-TET3mut (TET3mut) were treated with CHX for 4h or 8h. Whole cell lysates were subjected to western blotting (upper panel). Protein levels of TRα1 were detected by anti-GS antibody. The intensity of TR signals corrected by tubulin signals were plotted on the lower panel as 100% was set for the intensity measured for CHX 0h exposure. (D) Ubiquitination pattern of TRα1 in the presence or absence of TET3mut.
Whole-cell extracts were prepared from HEK293T cells transfected with indicated plasmids. Cells were treated or not with MG132&E64D before collection. Immunoprecipitates were obtained using M280 beads, half of the immunoprecipitated TRα1 was subjected to western blotting as loading control, while the other half was used to determine the ubiquitination level of TRα1 by incubating with an anti-ubiquitin antibody. The smear indicates the poly-ubiquitination of TRα1.

Fig.S3 SiTET3 mediated knockdown of TET3 decreased TRα1 protein level



(A) Expression levels of TET1-2-3 and TRα1 after siTET3 mediated knockdown. RNA were extracted from C17.2GSα cells transfected with siRNA against TET3 (siTET3) or negative control siRNA (siNC). Expression levels of indicated genes were examined by relative RT-QPCR. The expression levels were normalized to the level of 36B4 mRNA, with the amount of TET3 in siNC transfected cells set to 1. (B) Expression levels of *Epas1* and *Klf9* after TET3 knocking down in C17.2GSα cells. As outlined in (A), RNA were extracted from C17.2Gsα cells transfected with indicated siRNA, treated or not with T3 (10^{-8}M) for 24h before collection. Expression levels of indicated genes were examined by relative RT-QPCR. The expression levels were normalized to the level of 36B4 mRNA, with the amount in the siNC transfected C17.2-GSα (Veh) set to 1. (C) TRα1 protein level changes after TET3 knocking down in C17.2GSα cell .C17.2GSα cells were transfected with indicated siRNA, cells were pre-treated or not with MG132&E64D before collection. Whole cell lysates were subjected to western blot and TRα1 was detected with anti-GS antibody. Actin serves as a loading control.

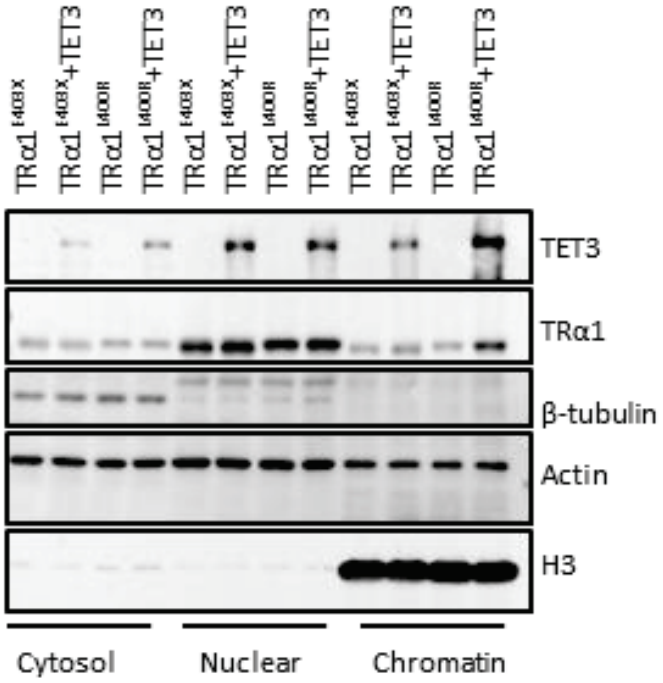
Fig.S4 Characterization of C17.2SαKO cells



(A) Scheme of CRISPR strategy and mutation identification.

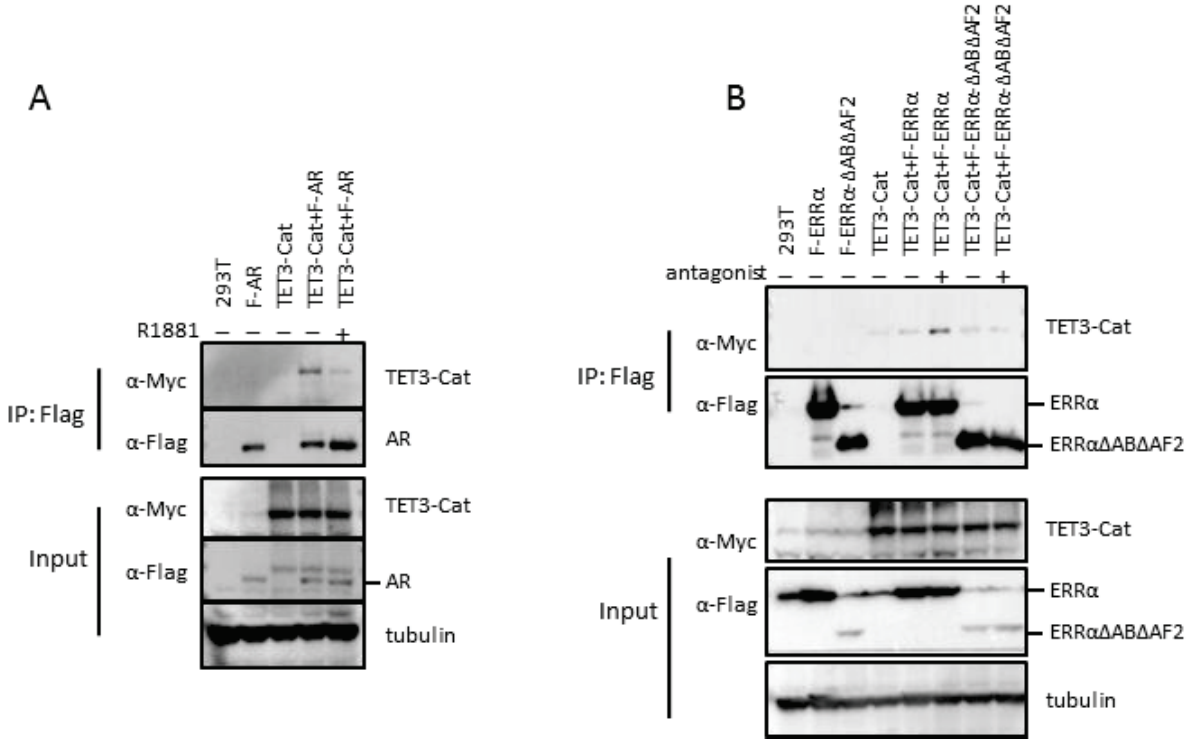
The top line represents the sequence of 21bp CRISPR guiding RNA used for genomic editing (underlined) to delete TET3 followed by an XGG PAM motif necessary for CRISPR action. Sequences below are the DNA sequencing results of the two alleles of TET3 in TET3 knockout C17.2Sα clone (C17.2SαKO). Two different frame shifts were successfully introduced on the two alleles. (B) Expression level of SBP-TRα1 and endogenous TET3 in control (C17.2SαC) and TET3KO (C17.2SαKO) cells measured by RT-QPCR. RNA were extracted from indicated cells treated or not with T3 (10^{-8}M), expression level of SBP-TRα1 and TET3 were examined by relative RT-QPCR. Expression levels were normalized to the level of 36B4 mRNA, with the amount in the C17.2SαC (Veh) set to 1. (C) Estimation of the efficiency of lentivirus infection in C17.2SαKO by immunofluorescence. C17.2SαKO cells were infected with lentivirus encoding human TET3 and RFP. RFP positive cells theoretically express human TET3. Immunostaining was performed as described in supplementary *Materials and Methods*.

Fig.S5 TET3 facilitates chromatin recruitment of TRα1^{L400R} but not TRα1^{E403X}



Effect of TET3 over-expression on the subcellular distribution of TRα1^{E403X} or TRα1^{L400R}. GS-TRα1^{E403X} (TRα1^{E403X}) or GS-TRα1^{L400R} (TRα1^{L400R}) were co-transfected with or without Flag-TET3 (TET3) in HEK293T cells, cells were fractionated using NE-PER nuclear and cytoplasmic extraction reagents kit. Cytosol, nucleus and chromatin fractions were subjected to western blotting. TET3 and TRα1 were detected by anti-Flag and anti-GS antibodies respectively. β-tubulin, actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin.

Fig.S6 TET3 interaction with other members of Nuclear Receptors



(A) Hormone modulated interaction between AR the androgen receptor (AR) and TET3. Myc-TET3-Cat (TET3-Cat) and Flag-AR (F-AR) were transfected in HEK293T cells. Cells were treated with or without R1881[1] (a synthetic agonist of AR at 10^{-7}M) before collection. Immunoprecipitaion was performed using M2 beads, co-immunoprecipitated TET3-Cat was detected by western blotting using anti-Myc antibody. (B) Antagonist modulated and AF2-domain dependent interaction between ERRα and TET3. Myc-TET3-Cat (TET3-Cat) and Flag-ERRα (F-ERRα) or a truncation mutant of ERRα without A/B and AF2 domain (F-ERRαΔABΔAF2) were transfected in HEK293T cells. Cells were treated or not with an antagonist (XCT790[2], 2.10^{-5}M) for ERRα before collection, immunoprecipitation was performed using M2 beads, co-immunoprecipitated TET3-Cat was detected by western blotting using anti-Myc antibody.

Supplementary Table 1: Primers for RT-QPCR

Primers for RT-QPCR

Genes	Forward	Reverse
Epas1	TGGACATCCCCCTGGACAGCAA	GGTCATGTTCTCCGAATCCAGGGCA
Klf9	ACATCGGGGAGAATGGGTGGGA	TTGTCCAACGAGCGCCAGACAC
36B4	ACCTCCTTCTTCCAGGCTT	CCCACCTTGTCTCCAGTCTTT
SBPTRα1	CTAGCGGCCATCAAACAAGT	CTGTTCTCCTCTGGGTCTGA
TET1	GCTGGATTGAAGGAACAGGA	GTCTCCATGAGCTCCCTGAC
TET2	GTCAACAGGACATGATCCAGGAG	CCTGTTCCATCAGGCTTGCT
TET3	ACTCATGGAGGATCGGTATG	GCTTCTCCTCCAGTGTGTGT

Reference:

1. Kemppainen JA, Lane MV, Sar M, Wilson EM. (1992) Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem* 267(2):968-74.
2. Willy PJ, Murray IR, Qian J, Busch BB, Stevens WC Jr, Martin R, Mohan R, Zhou S, Ordentlich P, Wei P, Sapp DW, Horlick RA, Heyman RA, Schulman IG. (2004) Regulation of PPARgamma coactivator 1alpha (PGC-1alpha) signaling by an estrogen-related receptor alpha (ERRalpha) ligand. *Proc Natl Acad Sci* USA. 101(24):8912-7.

Supplementary data for revision

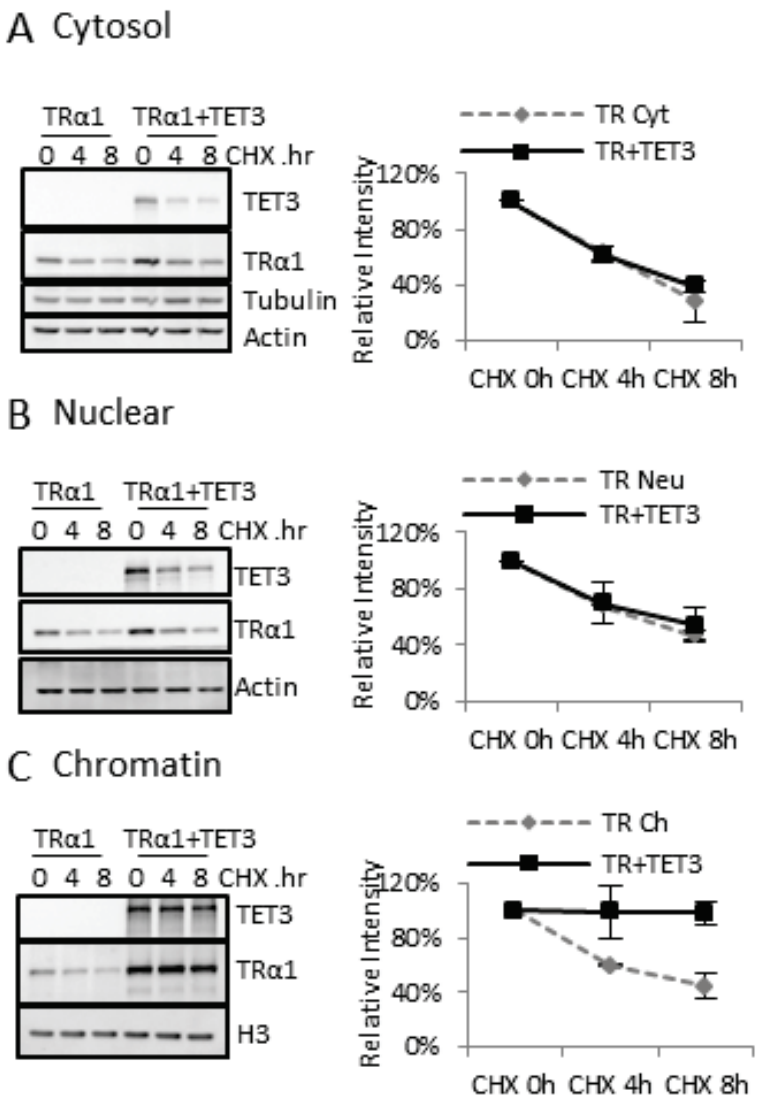
The paper that presents the TET3/TR study is under revision in PNAS at the time I am writing my thesis. we generated some supplementary results answering the reviewer's questions. I present these data here, since it allows clarifying several points of our study.

In the first version of our paper, we identified TET3 as a novel interactor for TR. This interaction has three consequences: TET3 stabilizes TR by protecting it from ubiquitination mediated degradation; TET3 facilitates the recruitment of TR to chromatin; TET3 increases the transcriptional activity of TR. The modulation effects observed here is independent of T3 treatment and the hydroxylase activity of TET3. Thus, we concluded that TET3 acts as an unconventional coactivator for TR. By stabilizing the protein and enhancing the chromatin binding of TR, TET3 could positively regulate the transcriptional capacity of TR. Based on the results, we proposed two different working models: 1) TET3 could increase the total amount of TR in the cells, thus more TR is present in the chromatin fraction; 2) TET3 could help tethering TR to the chromatin, the chromatin bound fraction of TR is stabilized by TET3. The increased level of chromatin bound TR increases the sensitivity of the cells to T3 treatment. However, we didn't manage to obtain a TR mutant which cannot be stabilized by TET3 or a mutant which lost its chromatin association ability, thus we failed to decipher between the two hypothesis.

To uncover the causal link of the two modulation effects of TET3 on TR, we examined the stabilization effect of TET3 on TR in different cell fractions in transfected HEK293T cells by adding cycloheximide (CHX). The protein level of TRα1 decreased over time upon CHX treatment. Addition of TET3 prevented the degradation of TRα1 in the chromatin fraction, but neither in cytosol nor in nuclear fraction, even though in the latter fraction both proteins are present (Figure.S7). This result show that TET3 stabilizes the chromatin bound fraction of TRα1, indicating that the chromatin association is a prerequisite of TET3 mediated stabilization of TR. However, this effect of TET3 on TR in chromatin fraction does not need the DNA binding ability of TR, since the DNA binding mutant of TR (TRα1G75S) is still chromatin bound and stabilized by TET3 (Figure.S8).

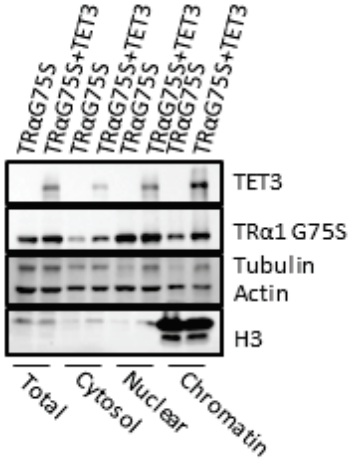
Another important observation in the initiation version of the paper is the potential role of TET3 in modulating the dominant negative effect of TRα1 mutants. We observed that different TRα1 mutants (exemplified with $TR\alpha1^{E403X}$ versus $TR\alpha1^{L400R}$ mutants) interact differently with TET3, this differential interaction with TET3 could determine the stoichiometry between mutant and WT receptors, and thus influence the dominant negative activity of the mutant receptor, and the disease severity. However, the difference in the effects of TET3 on $TR\alpha1^{E403X}$ versus $TR\alpha1^{L400R}$ presented in Fig.6D was quite modest. As the modest effect observed using luciferase assay is sensitive to both initial DNA amount and the expression efficiency of the different mutant transfected, we repeated the experiment with various ratios of mutant to wild-type TRα1 (Mutant/ TRα1). Specifically, we checked the effect of a fixed amount of TET3, on increasing ratio of Mutant/TRα1 and looking at the evolution of activity of a reporter gene induction upon T3 treatment. As expected, increasing the Mutant/ TRα1 ratio linearly decreases activity of wild-type TRα1, which is a reflection of increasing amount of mutant TRα1 leading to gradually enhanced dominant negative effect. More importantly, in the presence of TET3, the dominant negative effect of $TR\alpha1^{E403X}$ is less potent, displayed as a significantly diminished slope, whereas $TR\alpha1^{L400R}$ dominant activity is barely affected (Figure.S9).

Fig.S7 TET3 stabilize chromatin bound fraction of TRα1



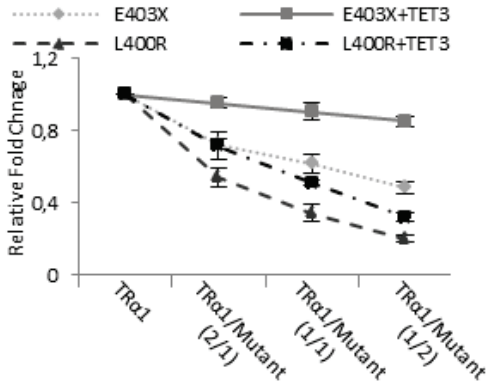
(A to C) Effects of TET3 on protein turnover of TRα1 in different cell fractions. HEK293T cells co-transfected with GS-TRα1 (TRα1) in the presence or absence of Flag-TET3 (TET3) were treated with CHX for 4h or 8h. After cell fractionation cytosol, nucleus and chromatin fractions were subjected to western blotting. TET3 or TRα1 were respectively detected by anti-Flag and anti-GS antibody. Tubulin, actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin. The intensity of TRα1 signals corrected by tubulin/actin or H3 signals were plotted on the right panel. 100% was set for the intensity measured in absence of CHX.

Fig.S8 TET3 enhances TRα1G75S recruitment to chromatin



Effect of TET3 over-expression on the subcellular distribution of the DNA binding mutant of TRα1 (TRα1G75S). GS-TRα1G75S (TRα1G75S) was transfected with or without Flag-TET3 (TET3) in HEK293T cells. After cell fractionation cytosol, nucleus and chromatin fractions were subjected to western blotting. TET3 or TRα1G75S were respectively detected by anti-Flag and anti-GS antibody. β-tubulin, actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin.

Fig.S9 Modulation of the dominant negative effects of TRα1^{E403X} or TRα1^{L400R} by TET3 in luciferase assay



HEK293T cells were transfected with DR4-luciferase, TRα1, varying ratios of TRα1/TR mutants (2/1; 1/1; 1/2) and TET3. Luciferase activities were measured 24 h after T3 (10^{-8}M) treatment. The relative fold change upon T3 treatment (taking the fold change of transfecting TRα1 alone as 1) was plotted on the graph. Each transfection condition was performed as triplicates, and the same experiments were repeated three times, error bars of the three independent experiments were indicated in the graph.

Project 2 (Work still in progress)

Title:

TET Family Proteins, New Modulators of RAR Mediated Transcriptional Regulation?

Abstract

TET proteins (TET1, TET2 and TET3) are newly discovered enzymes that can convert 5-methyl-cytosine (5mC) into 5-hydroxymethylcytosine (5hmC), acting as initiators for DNA demethylation process. Both 5hmC and the TET enzymes are abundant in embryonic stem (ES) cells. Combined deficiency of all three TETs led to depletion of 5hmC and deregulation of genes involved in ES differentiation. Retinoic acid (RA) is a well-known inducer of ES cells differentiation. Among the deregulated genes, a subset of RA response genes was identified, suggesting that RARs (retinoic acid receptors) and TETs might work together to regulate ES cell differentiation. The present study identified an interaction between TETs and RAR, this interaction is dependent on the DNA binding domain (DBD) of RAR and facilitates its recruitment to chromatin. Thus the deregulation of RA response genes after TETs' depletion is possibly a consequence of decreased RAR chromatin binding. Our results also suggest a role of TETs mediated 5hmC accumulation in modulating RAR chromatin accessibility. So this study evidences TET proteins act as regulators of RAR, modulating its access to chromatin rather that its intrinsic transcriptional activity, during RA induced differentiation of ES cells.

Introduction

DNA methylation, one of the best characterized epigenetic modifications in mammalian genome, is dynamically remodelled during the mammalian life cycle. After the establishment of DNA methylation pattern by de novo DNA methyltransferase DNMT3a and DNMT3b, the maintenance DNA methyltransferase DNMT1, together with its obligate partner UHRF1, recognizes the double strand DNA with hemimethylated CpG sites yielded by DNA replication, DNMT1 accomplishes the methylation of the CpG sites on the nascent DNA strand to ensure the maintenance of DNA methylation during cell devision (Bostick M et al, 2007). The Ten-eleven translocation (TET) enzymes (TET1, TET2, TET3) have been recently identified as key enzymes in regulating DNA demethylation process. TET enzymes share a C-terminal catalytic domain, which consists of a cysteine-rich domain (CRD) and a double-stranded beta-helix domain(DSBH), that catalyzes the hydroxylation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (Tahiliani et al., 2009). It has also been shown that TET proteins can further convert 5mC and 5hmC to 5-formyl cytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2011). Since 5hmC is poorly recognized by DNMT1/UHRF1, it has been proposed to promote passive DNA demethylation in a replication-dependent manner, alternatively, the oxidative 5mC serve as intermediates in active DNA demethylation where they are removed by the base excision repair machinery (Wu and Zhang, 2010; Branco et al., 2011). TET1 and TET3 also have a CXXC domain, which binds CpG-motives. The CXXC domain could be involved in regulating the genomic targeting of TET1 and TET3 (Xu Y et al, 2012; Tahiliani et al., 2009).

Both 5hmC and the TET enzymes are abundant in various embryonic cell types, including the zygote (Wossidlo et al., 2011), primordial germ cells (Hajkova et al., 2010) and embryonic stem (ES) cells (Ito et al., 2010). Both TET1 and TET2 are expressed in mouse ES cells, whereas TET3 is absent in ES cells and is

only induced upon differentiation, consistent with its ubiquitously distribution in various differentiated cell types (Dawlaty et al., 2013), in addition, TET3 is shown highly expressed in the oocytes and zygote (Gu et al., 2011). The recent generation of TET1, TET2, and TET3 single knockout as well as TET1/2 double-knockout (DKO) mice and ES cells has shed light on the roles of these proteins in embryonic and adult development. Depletion of either TET1 or TET2 reduces 5hmC levels but does not affect pluripotency. Both TET1 and Tet2 are dispensable for embryonic development, since adult mice are viable and fertile (Dawlaty et al., 2011; Koh et al., 2011; Li et al., 2011), while deletion of TET3 leads to neonatal lethality (Gu et al., 2011). More recent results showed that combined loss of TET1 and TET2 is compatible with embryonic development but promotes hypermethylation and compromises imprinting (Dawlaty et al., 2013). Combined deficiency of all three TETs depleted 5hmC and impaired ES differentiation. Global gene-expression and methylome analyses of TETs knockout EBs (embryonic bodies) revealed promoter hypermethylation and deregulation of genes implicated in embryonic development and differentiation. This suggests a requirement for TETs- and 5hmC mediated DNA demethylation for the proper regulation of gene expression during ES differentiation and embryonic development (Jaenisch R et al, 2014).

Accumulating evidences showed that TETs proteins possess catalytic activity dependent and independent gene regulatory functions. It was anticipated that they would contribute to gene activation by adjusting levels of DNA methylation at promoters. This was supported by the observation that both TET1 and 5hmC localize to transcriptional start sites (TSSs) (Williams K et al, 2011; Wu H et al, 2011). In addition, the enzyme activity of TETs has also been suggested to have a role in mediating transcriptional repression. TET1 genomic location is reported to overlap with the one of the Polycomb repressive complex PRC2. A stable interaction between TET1 and PRC2 could not be demonstrated, but TET1 might indirectly facilitate PRC2 chromatin binding by decreasing DNA methylation levels at PRC2 target genes (Wu H et al, 2011a; 2011b). However, their enzymatic activity independent transcriptional regulation has also been reported, TET proteins can serve as scaffolding proteins recruiting other proteins associated with transcriptional regulation. For example, TET1 interacts with the mSin3A complex (Williams K et al, 2011) and the MBD3/NuRD complex (Yildirim O et al, 2011). The two complexes are known to associate with histone deacetylases HDAC1 and HDAC2 enzymes that maintain histones in a deacetylated form, thereby repressing transcription. Thus TET1 could mediate transcriptional repression by directly recruiting the Sin3A/NuRD co-repressor complex to a subset of its target genes. Similarly, all three TETs were reported to interact with O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) to connect it to chromatin (Vella P et al, 2013). In turn, OGT would activate TETs target genes expression by glycosylating H2B and promoting subsequent trimethylation of histone H3 at lysine 4(H3K4me3), which is usually correlated with transcriptional activation (Shi FT et al, 2013; Deplus R et al, 2013).

Retinoic acid (RA), the main active vitamin A metabolite, is a well-known regulator of embryonic development as well as adult physiology (Mark M et al, 2006). The cellular effects of RA is mainly mediated by RARs (retinoic acid receptors) and RXRs (retinoic X receptors), which form heterodimers and act as RA-modulated transcription factors. RARs, including RARα, β and γ, belongs to nuclear receptor superfamily. RARs have a DNA binding domain and a ligand binding domain. RA addition triggers the binding to RARE (retinoic acid response element) of RAR/RXR hetreodimers, and the activation of RA response genes, such as Hoxa1, Cyp26a1 and RARβ2 (Gerard Benoit et al, 2015).

Coregulatory proteins, including co-activators and co-repressors, recruited to the RXR/RAR complex regulate the sensitivity of cells to RA's differentiation-inducing effects by modulating RAR activity. Among the various RA sensitive cell types, embryonal carcinoma (EC) and embryonic stem (ES) cells were shown to undergo differentiation upon RA stimulation (Mummery CL et al, 1990), and have been extensively used to study RAR activity. One of the most commonly used EC cell lines for the study of RA-dependent differentiation is F9 cells. In addition, though ES and F9 cells differentiate into different cell lineages upon RA treatment, studies showed that ES and F9 cells showed similar RA response, in terms of gene expression, at the early stage of differentiation (Mahony S et al, 2011; Gerard Benoit et al, 2015). Thus, characterized by their self-renewal capacity as well as their ability to differentiate into various cell lineages, ES and F9 cells provide invaluable biological models to study early developmental processes.

In this study, we identified a subset of RA responsive genes that are deregulated after TETs depletion in ES cells, suggesting a role of TET proteins in regulating RAR activity during RA induced ES differentiation. In addition, this deregulation is correlated with a decreased RAR binding on the RARE regions of some affected genes. Moreover, a RA induced 5hmC accumulation was observed on RARE of these deregulated targets, indicating an involvement of TETs enzyme activity in regulating their expression during ES differentiation. Further experiments need to be done to fully understand the mode of action of TET proteins in regulating RAR activity.

Results

Deficiency of TET proteins leads to deregulation of a subset of RA response genes during differentiation

By comparing the gene-expression pattern in TETs triple knockout embryonic bodies (TKO EBs) and WT EBs, derived from relevant ES cells, Jaenisch R et al. identified 1081 deregulated genes, indicating an important role of TET proteins in ES differentiation and development (Jaenisch R et al, 2014). By overlapping the 3971 genes, exhibiting significant differential expression after RA treatment in F9 cells (Benoit G et al, 2015), with the 1081 deregulated genes after TETs knocking out in ES, we obtainned 505 intersected genes (Fig.1A), implying a potential role of TETs in regulating specific gene expressions during RA induced differentiation of ES cells. Some significantly affected genes were chosen to further confirm the attenuated induction by RT-QPCR at the early stage of RA induced differentiation of ES cells (Fig.1B). Two known RA responsing genes, Cyp26a1 and RARβ2, which are not deregulated in TET TKO EB, showed similar expression profile in TKO and WT ES cells upon RA treatment (Fig. 1B). Thus, TETs influence some but not all genes in respond to RA.

TET proteins interact with RAR

RARs mediate most of the actions of RA in stem cells (Gudas LJ et al, 2011). As TET3 has been shown to interact with and regulate the transcriptional activity of TR, another nuclear receptor, we checked whether TET proteins could interact with RARγ, which is one abundant RAR isoform in ES cells. Co-immunoprecipitation assays were performed for TETs and RARγ in HEK293T cells. As full length TET proteins are difficult to produce, the catalytic domains (TET-Cat) were used as a first intention. All three TETs displayed interactions with RARγ. However, as RARγ interaction with TET3-Cat was the strongest (Fig.2A), the interactions with TET1-Cat and TET2-Cat were considerably weaker, and the three TETs

share high homology in structures, future studies were only conducted with TET3. Then the interaction of RAR with full-length TET3 was also validated (Fig.2B), this interaction is not modulated by RA.

TET3 and RAR interacts via the CXXC & catalytic domains in TET3 and DBD domain in RAR

To more precisely map the interaction domains within the two proteins, a series of vectors expressing tagged and truncated TET3 (Flag) or RARγ (GS) were generated (Fig.3A). Co-immunoprecipitation assays showed that both the N-terminal CXXC domain and the C-terminal catalytic domain of TET3 interact independently with RARγ. Moreover, within the catalytic domain of TET3, both the DSBH (double strand β-helix domain) and the preceding CRD (cysteine-rich domain) show interactions with RARγ. Conversely, the DNA binding domain (DBD) within RARγ is responsible for the interaction with TET3 (Figure.3B and 3C).

TETs modulate RA response by regulating chromatin association of RAR

Given that TET3 interacts with the DBD of RAR, we first tested the possibility that TET proteins may influence RAR recruitment to its target genes. Cell fractionation of transfected HEK293T cells confirmed that RAR is mainly recovered in the nucleus, but only a small sub-fraction is chromatin bound. Presence of TET3 substantially increased the chromatin bond fraction of RAR (Fig.4A). Moreover, ChIP assays for RAR in TKO ES and WT ES cells further confirmed the positive role of TETs in facilitating RAR recruitment to its response element. Depletion of TET proteins decreased RAR binding at the RARE regions of deregulated target genes, such as Cyp26b1, Tshz1 and Hoxb9 (Fig.4B), while TETs' knocking out has no visible effects on RAR binding to unaffected target gene promoters, exemplified by Cyp26a1 and RARβ2 (Fig. 4B). This decreased binding of RAR to specific targets is not due to the decreased RAR protein level after TETs' depletion (Fig.S1A and S1B).

5hmC accumulates at RARE of specific deregulated genes in WT ES upon RA treatment

In addition, TETs/RAR interaction could influence the DNA methylation status near RAR binding sites, thereby contributive to regulating RAR target gene expression. To investigate whether the enzyme activity of TETs is involved in regulating the expression of those deregulated target genes, we performed a preliminary test checking the 5hmC level at RAREs of the three deregulated RAR target genes by hMeDIP assay. We observed folds accumulation of 5hmC on RAREs of Cy26b1 and Tshz1 upon RA treatment in WT ES cells. However, the 5hmC level on RARE of Hoxb9 is unchanged (Fig.5). Same experiments were done for two unaffected genes after TETs' knocking in ES, Cyp26a1 and RARβ2, 5hmC level did not show any difference with or without RA treatment (Fig.5).

Discussion

In this study, we demonstrated that depletion of TET proteins leads to deregulation of a subset of RAR target genes during RA induced differentiation of ES cells. This deregulation is possibly a consequence of decreased RAR binding on the RARE region of these genes after TETs' knocking out. An interaction between TETs and RAR was observed by co-immunoprecipitation assay, and the CXXC and catalytic domain in TETs interacts independently with the DBD in RAR, over-expression of TETs facilitated RAR recruitment to chromatin. Although other possibility exists, a simple hypothesis would be that, by interacting with RAR, TET proteins help tethering RAR to the RARE region of a subset of RA response

genes, thereby increasing their expression during RA induced differentiation. Based on the present results, we cannot exclude the possibility that TET proteins facilitate the recruitment of other proteins, other than RAR, which are necessary for the RA regulation of these genes. The specific genomic targeting of TET proteins might explain why only a subset of genes is influenced by TET protein. Thus studies allowing to assessing the dynamic binding of TET1/2 and TET3 in differentiating ES at the whole genome scale would shed light on this issue.

The accumulation of 5hmC level observed at RARE of Cy26b1 and Tshz1 upon RA treatment in WT ES cells raises the possibility that the deregulation of these two genes in TKO ES may due to the depletion of 5hmC after TETs' knocking out. Thus the DNA demethylation activity of TETs may have a role in modulating RAR binding and/or its transcriptional activity towards these two targets. To further support this hypothesis, the DNA methylation level (5mC) on RAREs of these genes should be compared in WT and TKO ES cells. Moreover, monitoring the rescue effect on the expression profiles of these genes of enzymatic dead mutant of TET proteins in TKO ES cells could also help answer this question.

In contrast, 5hmC level on the RARE region of Hoxb9 does not change after RA treatment, even though its expression is similarly affected by TETs depletion. One possible explanation for this difference would be that we are not looking at the RARE region responsible for Hoxb9 activation, since Hox genes are organized in clusters on the genome and different RAREs are located in this cluster area. In addition, the expression profile of the three deregulated target genes, Cyp26b1, Tshz1 and Hoxb9, are actually different. Hoxb9 exhibits an earlier RA activation, while the other two genes showed a delayed activation pattern. A previous study showed that a subset of RAR target genes displayed an increase in PRC2 recruitment simultaneously with transcriptional activation in response to RA. This RA induced enrichment of PRC2 resulted in attenuation of the maximum transcriptional activation, allowing for a relative delayed induction by RA (Laursen KB et al, 2013). However, how PRC2 is recruited to this specific subset of target genes is still elusive. Another study showed that TET1 might indirectly facilitate PRC2 chromatin binding by decreasing DNA methylation levels at PRC2 target genes (Wu H et al, 2011a; 2011b). Thus it is reasonable to propose that the accumulated 5hmC marks on RARE located in Cy26b1 and Tshz1 promotes PRC2 recruitment, which leads to their delayed induction by RA. Thus the TETs mediated 5hmC dynamics may serve as a distinguishing feature between two classes of RA-inducible stem cell genes, such a mechanism would permit the fine-tuning of transcriptional networks during differentiation. Further experiment monitoring the PRC2 recruitment and/or H3K27me3 (catalyzed by PRC2 complex) level on the RARE regions of Cyp26b1 and Tshz1 would help addressing this hypothesis.

We observed that all three TETs are able to interact with RARγ, with the most salient interaction observed for TET3/RARγ. Even though we focus our study on TET3/RARγ, we had demonstrated a weaker but reproducible interaction between RAR and the other two TETs. As the three TETs are expressed during ES differentiation, with TET1 and TET2 highly expressed in ES cells and down regulated during differentiation and TET3 expression appearing only upon differentiation (Dawlaty et al, 2013), one can wonder whether they all or specifically modulate the RA response. It is highly possible that TET1/2 contribute to RAR activity at the early stage of RA induced differentiation, while TET3 would fine-tune RAR mediated differentiation at a later stage. This question could be better answered using

TET1/2/3 single knock out ES cells, or by reintroducing each TET protein into the TKO ES and studying RA response.

We previously described a physical interaction between TET3 and TR (thyroid hormone receptor). TET3 enhance the cell sensitivity to thyroid hormone treatment by enhancing the stability of TR in the chromatin fraction. This modulation effect relies on a direct interaction between the two proteins and does not require the demethylase activity of TET3. The interaction with TET3 was also observed for AR (androgen receptor) and ERR (estrogen receptor related receptor), implying that TET proteins are general modulators for NRs. The nature of the interaction between TET3 and RAR identified in this study is different, since it involves the DBD in RAR, instead of the LBD in TR, implying a different mode of action of TET3 in modulating RAR transcriptional activity. Studies in ES (Mahony S et al, 2011) and F9 (Gerard Benoit et al, 2015) cells showed that the most evident consequence of RA stimulation is a massive increase in the number of RAR/RXR bound genomic loci, indicating that the increased RAR chromatin binding is a major effect of ligand-dependent activation. In the present study, TET proteins has no visible effect in modulating RAR protein level, instead, TET proteins contribute to activate a subset of RA response genes by facilitating RAR recruitment. Whether the enzyme activity of TET proteins are also involved in this modulation effects is still elusive for the moment. The exact mechanisms involved in the regulation of RAR transcriptional activity by TET proteins remains to be found. Having both similarities and differences, it is possible that the modulation effect of TET3 towards different nuclear receptors is highly dependent on the milieu of cofactors and/or the specific cellular contexts.

Materials and Methods

Cell culture and DNA transfection

TKO ES cell line was a kind gift from Prof. Jaenisch Rudolf (Jaenisch R et al, 2014). ES cells were expanded on feeders using regular ES media containing leukemia inhibitory factor (LIF). Human HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. These cells were grown at 37 °C in 5% CO_2. We used *Trans*IT®-LT1 Transfection Reagent (Mirus) for transfection according to the manufacturer's instructions.

Plasmids and Antibodies

Flag-tagged TET1, TET2 and TET3 constructs were generated as previously described (Zhang Q et al, 2014). Briefly, the full-length complementary DNAs (cDNAs) of human TET1, TET2, TET3 were constructed by ligating various fragments amplified by PCR from the human H9 ES cells and then cloned into pcDNA3.1-Flag vector to express Flag-tagged proteins. Deletion mutants TET3 were generated by PCR amplification and then cloned into p3xflag-cmv-9. The enzymatic dead mutant of TET3 ((H1077Y/D1079A)) in p3xflag-cmv-9 was created by PCR-directed mutagenesis. TET3 deletion mutants were generated by PCR amplification and cloned into p3xflag-cmv-9 (Sigma). All plasmids were verified by DNA sequencing. The antibodies used were: monoclonal anti-FLAG® M2-Peroxidase (HRP) antibody (A8592,Sigma), monoclonal anti-β-Actin (A5316,Sigma), anti-GFP (ab290,Abcam), anti-beta-tubulin (ab6046, Abcam), anti-RAR (SC-773, SantaCruz) and anti-Histone3 (ab1791, Abcam).

RNA Extraction and qPCR Measurements

Total RNA was extracted from cells with the Macherey-Nagel RNA II kit according to manufacturer's recommendations. RNA quantities were measured with a Nanodrop (Thermo Scientific) spectrophotometer before reverse transcription. One microgram of each RNA samples was reverse transcribed using M-MLV reverse transcriptase (Promega). Quantitative PCRs were then performed in 96-well plates, using the SYBRGreen mix (BioRad iQ supermix), Expression levels were calculated using the $2^{-\Delta\Delta(Ct)}$ method.

Immunoprecipitation

For immunoprecipitation, HEK293T cells were lysed with the lysis buffer (50mM Tris-HCl pH 7.5, 150mM KCl, 1% Triton X-100,1mM EDTA, 8% glycerol). The cell lysates were cleared by centrifugation at 14000rpm for 10 min at 4 ℃. Supernatants were diluted in IP binding buffer (50mM Tris-HCl pH 7.5, 150mM KCl, 0,1% Triton X-100,1mM EDTA, 8% glycerol), and incubated with magnetic M2 beads (Sigma)or magnetic M280 beads (Dynabeads® M-280, Invitrogen) at 4℃ for 3 hrs. Beads were then washed four times with wash buffer (50mM Tris-HCl PH7.5, 150mM KCl, 0.1% Triton X-100, 1mM EDTA) and then boiled in 1X SDS loading buffer. The eluted precipitates were subjected to SDS-PAGE and western blotted with the indicated antibodies.

Cell Fractionation

Nuclear–cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, HEK293T cells transfected with indicated plasmids were collected and washed with PBS, then cells were re-suspended in buffer CERI (cytosol extraction buffer I), and incubated on ice for 10 minutes, CERII (cytosol extraction buffer II) were added and cytosol fraction were collected by taking the supernatant after high speed centrifugation. The insoluble fraction was washed with CERI, and the pellet was treated with NER (nuclear extraction buffer) and incubated on ice for 40min with intervallic vortex, the supernatant (nuclear fraction) was collected after high-speed centrifugation. After washing the pellets with buffer NER, the insoluble material was solubilized in chromatin extraction buffer (20 mM Tris-HCl, 10 mM EDTA, 500 mM NaCl and protease inhibitor, pH 7.9) by sonication.

Chromatin immunoprecipitation (ChIP) assay

ChIP experiments were performed as previously described (Gerard Benoit et al, 2015). Briefly, cells were cross-linked by a two-step procedure, with 2mM disuccinimidyl glutarate (Pierce) for 30 min at room temperature and then with 1% formaldehyde (Sigma- Aldrich) for 10 min at 37◦C. After lysis, chromatin extracts were sonicated to reduce the length of DNA fragments to 200-600 bp using a Diagenode Bioruptor. Antibodies anti-panRAR (Sc-773, Santa Cruz Biotechnology) was used to immunoprecipitate RAR-bound DNA fragments. Immunoprecipitated chromatin were analyzed by quantitative real-time PCR, the results were normalized with respect to input.

Locus-specific 5hmC analysis (hMeDIP)

To immunoprecipitate 5hmC, genomic DNA was extracted from indicated ES cells, purified genomic DNA was sonicated to 200–1000 base pairs (bp) and heat-denatured (10 min, 95°C). An aliquot (1ug) of sonicated genomic DNA was saved as input. Four micrograms of fragmented genomic DNA was immunoprecipitated with 4ug of rabbit 5hmC Ab (Active Motif, catalog no. 39791) overnight at 4°C in a final volume of 500 uL of immunoprecipitation buffer (10 mM sodium phosphate at pH 7.0, 140mM NaCl, 0.05% Triton X-100). The DNA–antibody mixture was incubated with 40uL of protein G Dynabeads (Invitrogen) for 2 h at 4°C and washed three times with 1 mL of immunoprecipitation buffer. The beads were then treated with proteinase K for at least 3 h at 55°C, and the precipitated DNA was purified and subjected to Q-PCR analysis using SYBR Green (Invitrogen).

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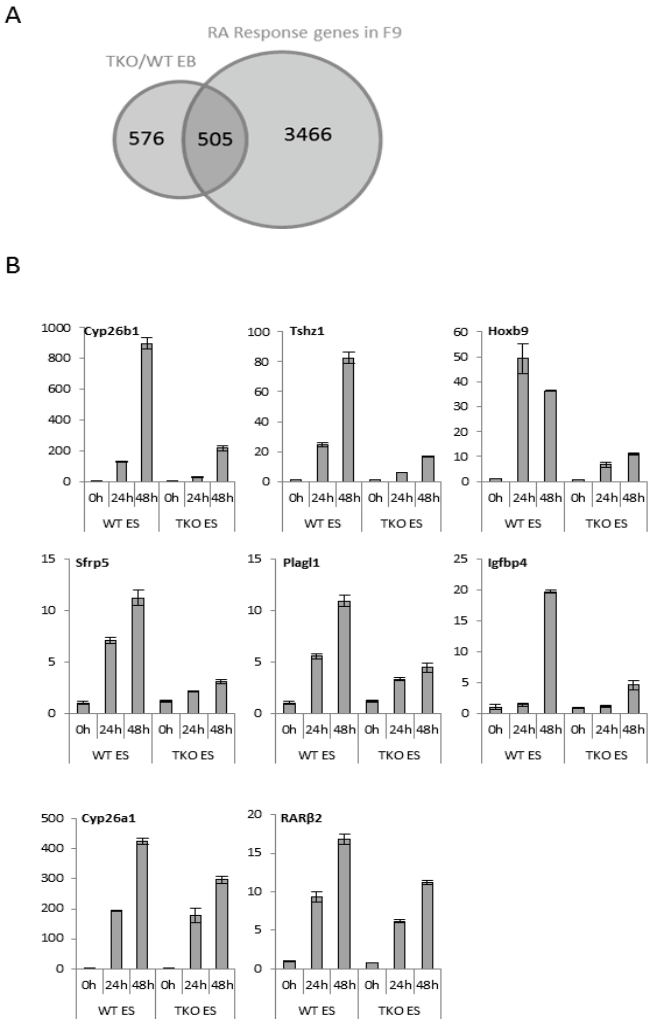
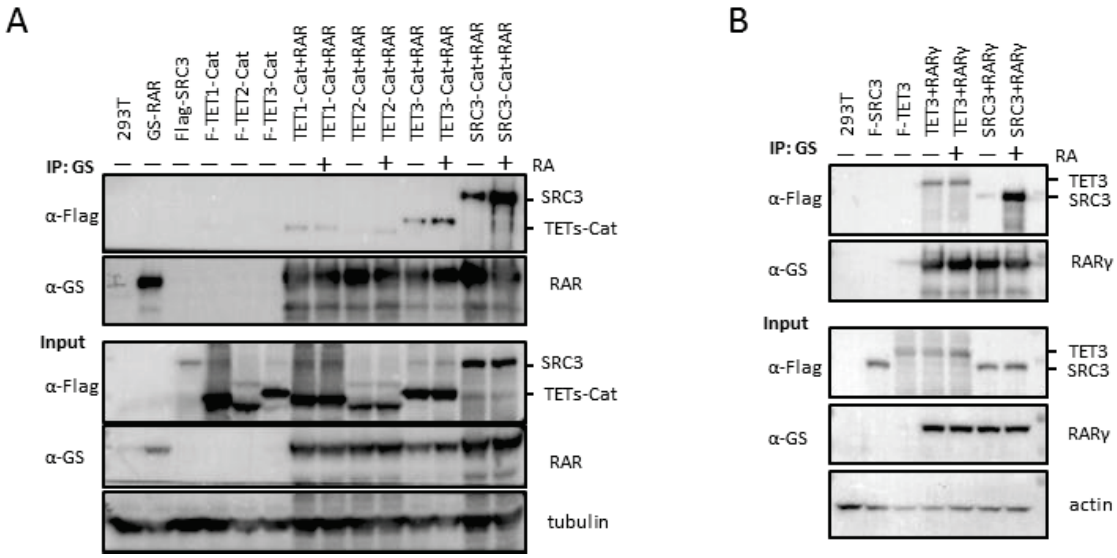
Figures and Figure legends

Figure.1 Identification of deregulated RA response genes after TETs' knocking out



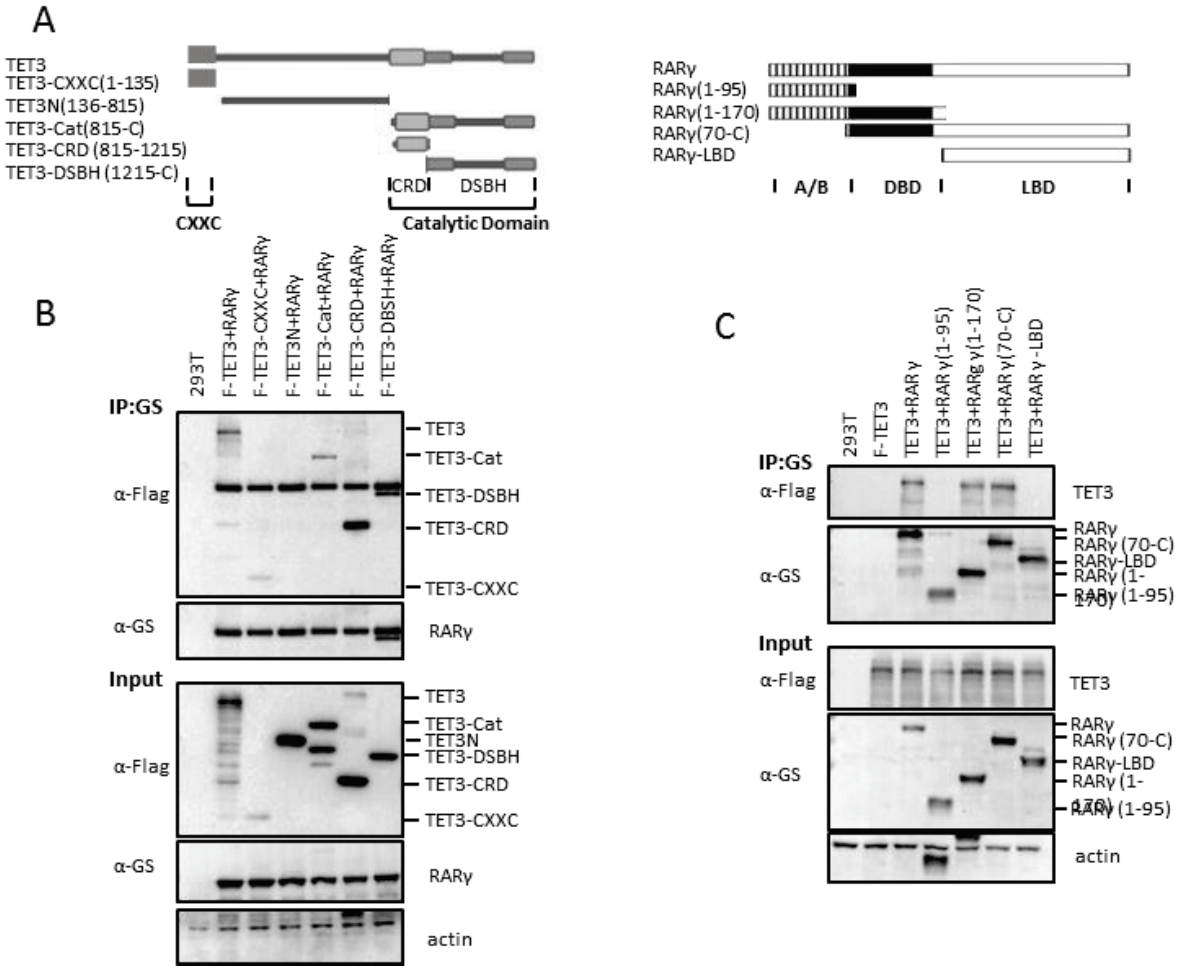
(A) Intersected genes between genes deregulated after TETs' knocking out in ES and RA responsive genes in F9 cells. An overlap was made between two subsets of regulated genes: deregulated genes in TKO EB compared to WT EB (1081); RA responsive genes in F9 cells upon RA treatment (3971). 505 genes were present in both lists of genes. (B) Expression profiles of selected overlapping genes in WT vs TKO ES cells upon RA treatment. RNAs were extracted from Indicated cells: control cells (WT ES), TETs KO cells (TKO ES). Cells were treated or not with RA (10^{-6}M) for indicated time, expression level of selected overlapping genes were examined by relative RT-QPCR. Expression levels were normalized to the level of 36B4 mRNA, with the relative expression in WT/0h arbitrarily set to 1.

Figure.2 Identification of an interaction between TETs and RARγ



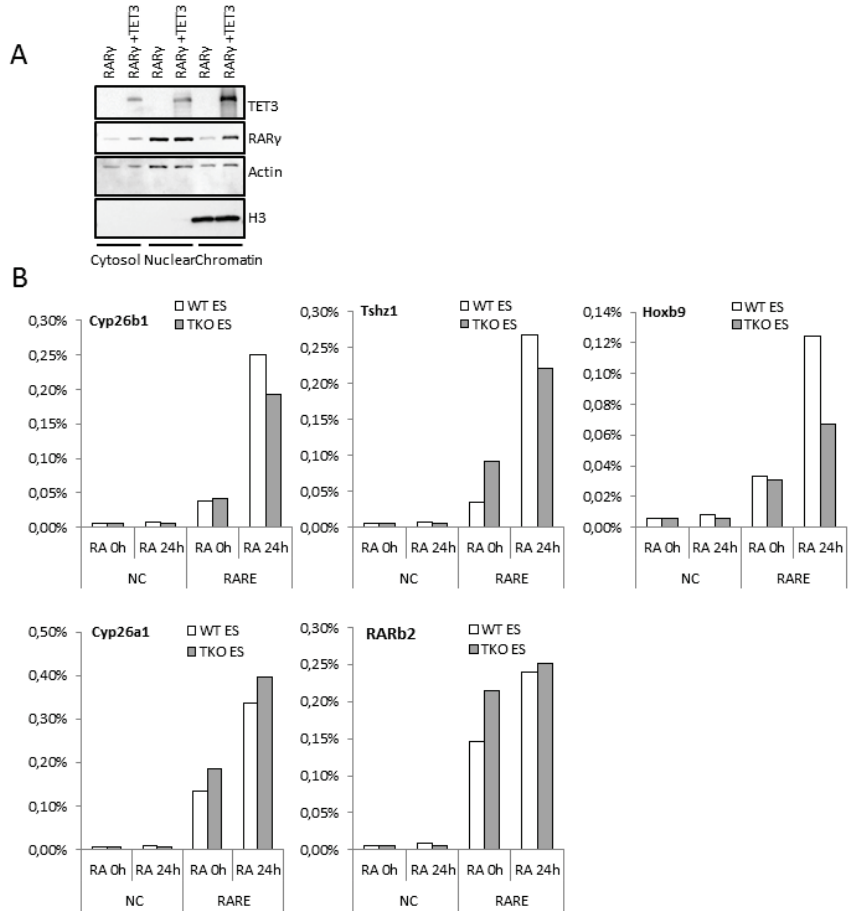
(A) Interaction between GS-RARγ and Flag-tagged catalytic domain of TETs (F-TETs-Cat) or SRC3(F-SRC3) identified by co-immunoprecipitation assay. Whole-cell extracts were prepared from HEK293T cells co-transfected with GS-RARγ and F-TETs-Cat or F-SRC3, treated or not with RA (5.10^{-8} M) before collection. Immunoprecipitates were obtained using M280 beads that retain GS tag. Co-precipitated TETs-Cat or SRC3 were detected by western blotting using anti-Flag antibody. SRC3 was used as a positive control for all the ensuing co-immunoprecipitations as RAR/SRC3 interaction is well-known to be RA dependent. Tubulin serves as a loading control. (B) Interaction between GS-RARγ and Flag-tagged full-length TET3 validated by co-immunoprecipitation assay. Flag-TET3 or Flag-SRC3 and GS-RARγ were transfected in HEK293T cells and immunoprecipitated using an M280 beads , co-immunoprecipitated TET3 or SRC3 was detected by western blotting using anti-Flag antibody. Actin serves as a loading control.

Figure.3 The CXXC and catalytic domains of TET3 and the DBD domain of RARγ conferred the interaction between the two proteins.



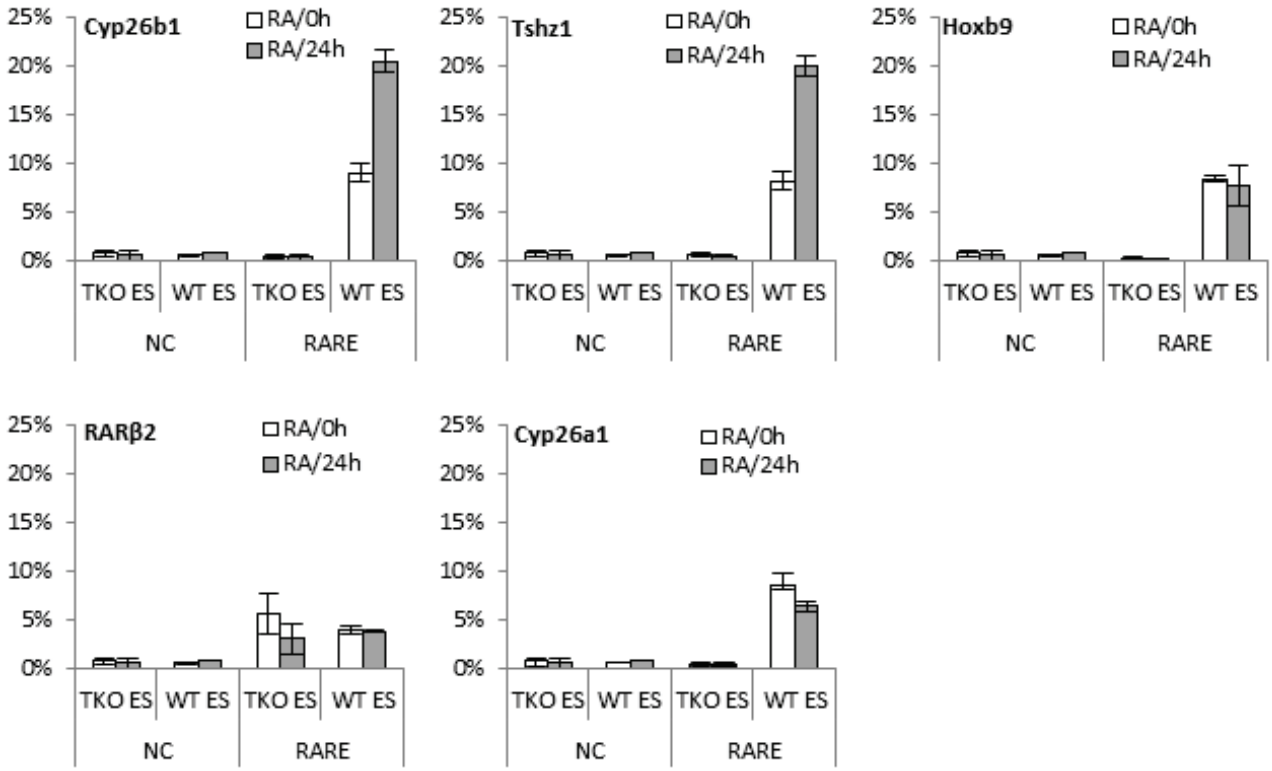
(A) Schematic representation of full-length and various truncation mutants of TET3 and RARγ. CXXC, CXXC domain; CRD, Cat, catalytic domain; cysteine-rich domain; DSBH, double-stranded beta-helix domain; DBD, DNA binding domain; LBD, ligand binding domain. (B and C) Co-immunoprecipitation assays identifying the interacting regions between TET3 and RARγ. Whole-cell extracts were prepared from HEK293T cells co-transfected with indicated plasmids. (B) GS-RARγ was precipitated with M280 beads, co-precipitated F-TET3 mutants were detected using Flag antibody. (C) M280 beads were used to precipitate RARγ and different truncation mutants, co-precipitated TET3 was detected with Flag antibody.

Figure.4 Interaction between TET3 and RARγ enhanced RARγ recruitment to chromatin



(A) Effect of TET3 over-expression on the subcellular distribution of RARγ. GS- RARγ(RARγ) were co-transfected with or without Flag-TET3(TET3) in HEK293T cells, cells were fractionated using NE-PER nuclear and cytoplasmic extraction reagents kit. Cytosol, nucleus and chromatin fractions were subjected to western blotting . Protein level of TET3 or RARγ were respectively detected by anti-Flag and anti-GS antibody. Actin and H3 were respectively the loading controls for the cytosol, nucleus and chromatin. (B) Effect of TETs knocking out on RAR recruitment to deregulated target genes. ChIP assays for RAR were performed using RAR antibody in both WT and TKO ES cells treated with RA for indicated time. RAR ChIP for the two genes, Cyp26a1 and RARβ2, which are not in the list of deregulated RA response genes after TETs' knocking out were also shown. A negative control region (NC) was the region 2-3Kb away from relative RARE region of indicated target genes. The results were presented as percentage of input.

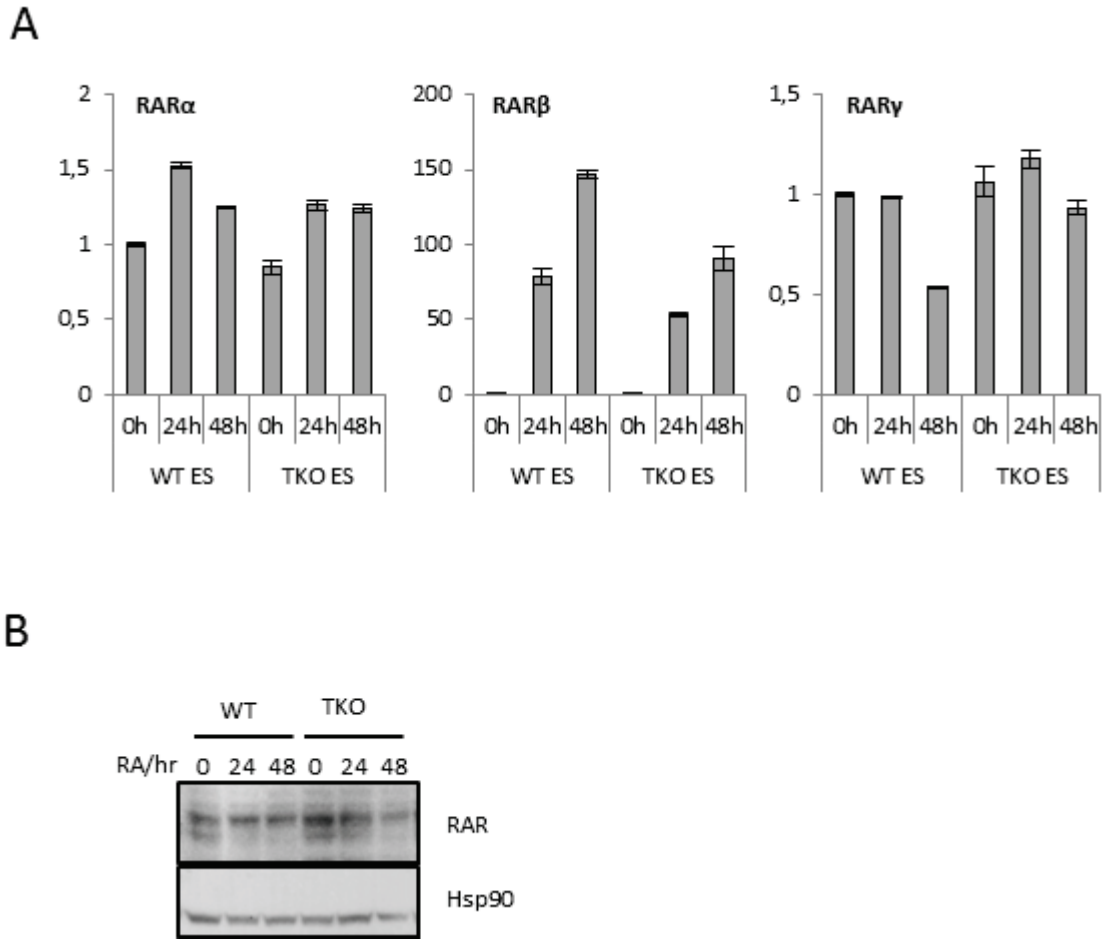
Figure.5 5hmC level on RARE regions of RAR target genes in WT and TKO ES upon RA treatment.



Genomic DNA was extracted from WT and TKO ES cells treated or not with RA for indicated time, 5hmC antibody was used to precipitate 5hmC modified DNA, then primers against NC (negative control region, 2-3 Kb away from RARE region) and RARE region of indicated genes were used for Q-PCR. The results were presented as percentage of input.

Supplementary data

Figure.S1 Effect of TETs knocking out on RAR mRNA and protein level.



(A) RNAs were extracted from Indicated cells: control cells (WT ES), TETs KO cells (TKO ES). Cells were treated or not with RA (10^{-6}M) for indicated time, expression level of selected overlapping genes were examined by relative RT-QPCR. Expression levels were normalized to the level of 36B4 mRNA, with the relative expression in WT/0h arbitrarily set to 1. (B) Total protein level of RAR was checked in both WT and TKO ES treated or not with RA for indicated time.

Table S1 Primers used in this study.

Primers for RTQPCR		
Gene	Forward	Reverse
Cyp26a1	GTCAGCAGTTGTCTGGAGCA	AGAGAAGAGATTGCGGGTCA
RARβ2	CCAGGTATACCCCAGAGCAA	GCCTGTTTCTGTGTCATCCA
Cyp26b1	TACCCAGGGCAAAGACTACT	GTAGGCTGCGAAGATCAACT
Hoxb9	GGCTACGGGGACAATAAAAT	CTAGCTCCAGCGTCTGGTAT
Tshz1	CCGAAATAGATGAGGAGCAC	ATAGCTCTGGGCTTCTTTGA
Igfbp4	AGACCTCTGACAAGGATGAG	CATCTTGCTCCGATCTCTAA
Sfrp5	GACAACGACCTCTGCATC	AGTCACTGGAGCACATCT
Plagl1	AAAGCCTTCGTCTCCAAGTA	CTTCCGGTTGAATGTCTTCT
RARα	AGATGGACGATGCTGAGACT	TCCGGACGTAGACTTTCAGT
RARβ	AGAAACAGGCCTTCTCAGTG	GTGGTTCTTGGAGCTTGTCT
RARγ	CCAGTACTGCAGGCTACAAA	GAGCCCTCCTCTTTTACCTC

Primers for ChIP		
Name	Forward	Reverse
Hoxb9-RARE	CCCACACCAAAGCTATTCTC	TGATCCCGGACTCTATGAAT
Hoxb9-NC	CGGTGCTTTTTATACCCAAC	GGTTTGCAACTGAAGGCTAT
Cyp26b1-RARE	CCCTCACCTCTGAATGAACT	GGAAAGCAAGCCACAATAAT
Cyp26b1-NC	CCACCACAGAGGTGTCATTA	AGTTGTAGCCTGGGTGAGAG
Tshz1-RARE	CTCAGCAGCAGGGGTTAGAG	AACCCAGACAAGCATGGAAG
Cyp26a1 RARE	TTCACTGAGATGTCACGGTCC	TTCCCAATCCTTTAGCCTGA
RARβ2 RARE	GGGAGTTTTTAAGCGCTGTG	CGGAGCAGCTCACTTCCTAC

General Discussion and Perspective

Proposed working models

Based on my thesis work, we proposed two working models of TET3 in modulating transcriptional activity of TR or RAR. 1) TET3/TR: TET3 interacts with the ligand binding domain of TR, this interaction has been shown to increase the chromatin association of TR by protecting the chromatin bound TR from ubiquitination-proteasome mediated degradation. The increased level of chromatin bound TR increases the sensitivity of the cells to T3 treatment. 2) TET3/RAR: TET3 interacts with the DNA binding domain of RAR, this interaction helps facilitating RAR recruitment to a subset of RAR target genes. The hydroxylase activity of TET3 would modulate the transcriptional activity of RAR. We hypothesized that TET3 contributes to RAR mediated transactivation by maintaining a hypomethylated state at target gene promoters. 5hmC (the main catalyzing product of TET3) would facilitate RAR binding to the promoters of its target genes and/or in distinguishing early and late RA-inducible stem cell genes (in cooperation with PRC2 complex).

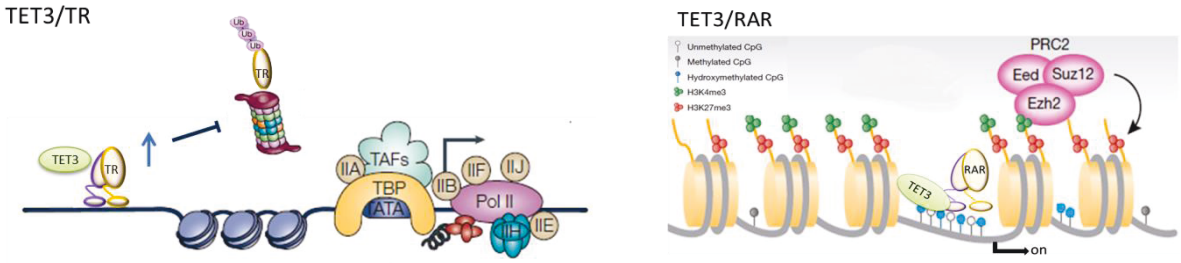


Figure 26 Working models for TET3 in modulating TR/RAR activity.

TET3/TR: TET3 interacts with the LBD domain of TR, this interaction could increase the TR presence on chromatin by protecting it from ubiquitination-proteasome mediated degradation. TET3/RAR: TET3 interacts with the DBD domain of RAR, this interaction facilitate RAR recruitment to some RAR target genes, the enzyme activity of TET3 may have a role in mediating RAR transactivation by modifying the micro-environment of the chromatin. The 5hmC deposited by TETs may serve as a distinguishing feature between early and late RA inducible genes by facilitating PRC2 complex recruitment.

Differences and similarities with respect to TET3/TR and TET3/RAR

We identified TET3 as a novel interaction partner for both TR and RAR. However, the functional consequence of the two interactions is similar but not completely identical.

Interactions

Both TR and RAR interact with TET3 independently of hormone treatment. The N-terminal CXXC domain and the TET3-Cat (C-terminal catalytic domain of TET3) confer its interaction with both receptors. However, the interaction surface varies from TR to RAR. Indeed, the AF2 domain (Helix 12) within the LBD (ligand binding domain) of TR is responsible for mediating its interaction with TET3, while the DBD (DNA binding domain) of RAR serves as the interaction surface with TET3. We did not however test the

importance of the C-terminal helix of RAR LBD, or the same DBD deletion in TR and RAR, so it could be that the mode of interaction is not as divergent as it appears at first sight.

Stabilization and recruitment to chromatin

TET3 was shown to enhance the chromatin association of both TR and RAR. TET3 increase the chromatin association of TR by inhibiting ubiquitination-proteasome mediated degradation of the chromatin bound TR. In addition, a compromised TR protein level was observed after knocking out TET3 in C17.2 cells. TET3 was also shown to facilitate RAR recruitment to promoters of some RAR target genes. However, depletion of TET proteins in ES cells has no obvious effect on total protein level of RAR. Since the experiments related to TET3/RAR were performed with different assays in different cell systems, we should be cautious to conclude that TET3 has no effect in stabilizing RAR. A similar CHX experiment with RAR in different cell fractions should be performed, as whole cell measurement may not be able to capture variations restricted to the chromatin fraction.

Activation of target genes

TET3 was involved in regulating the hormone response of a subset of genes in both cases. In the case of TET3/TR, TET3 regulates the T3 sensitivity of the cells mainly by regulating the protein turn-over of chromatin bound TR. Thus the expressions of genes, which are sensitive to TR protein level changes, are modulated by TET3 (exemplified by Epas1). For the moment, we have no results indicating that TET3 could stabilize TR on every target genes, thus a co-localization of TR and TET3 at promoters may be a precondition for a role of TET3 in modulating their expressions. A genome-wide study relating to TET3 and TR occupancy could help validating this idea. In the case of TET3/RAR, TET3 was involved in regulating a subset of RAR target genes by facilitating the recruitment of RAR to their promoters. Although the exact mechanism underline this facilitation effect is still elusive, the genomic targeting of TET3 may be involved in defining the regulated genes. Thus studies related to the dynamic binding of TET1/2 and also TET3 in differentiating ES cells would be of significance for this respect.

Involvement of hydroxylase activity of TET3

Our results suggested that the hydroxylase activity of TET3 is involved in regulating the expression of some RAR target genes, albeit the possible implication of the enzyme activity of TETs awaits further investigations. Inspired by previous studies, we proposed three possible ways of TETs' hydroxylase activity in regulating RAR target gene expression. Firstly, TET proteins could contribute to activate RAR target gene expression by removing the repressive DNA methylation (5mC) at their promoter regions. Examining the DNA methylation level (5mC level) on those gene promoters in differentiating ES cells may help validating this hypothesis. Secondly, 5hmC, the catalyzing products of TET proteins, could affect the DNA affinity of RAR, such as the case of TET1/PRC2 (Williams et al, 2011; Wu et al, 2011). Band shift assay examining the affinity between RAR and unmodified DNA/ 5mC modified DNA/ 5hmC modified DNA could help resolving this issue. Thirdly, TETs mediated 5hmC dynamics, together with PRC2 complex (Lauren et al, 2013), may serve as a distinguishing feature between early and late RA-inducible stem cell genes (exemplified by Cyp26b1 VS Hoxb9).

The modulation effect of TET3 on TR presented above is independent of its hydroxylase activity. Even so, we could not exclude the possibility that the hydroxylase activity of TET3 may contribute to the fine-tuning of its enzyme activity independent role in regulating TR target gene expression. Examining whether the enzymatic inactive mutant of TET3 could fully rescue the deregulation of TR target genes in TET3 knockout C17.2 cell lines (termed C17.2SαKO) may help to elucidate the specific role of TETs' enzyme activity in modulating TR transactivation. In addition, monitoring the DNA methylation (5mC) level and/or 5hmC level at the promoter region of TR target genes in C17.2 control (termed C17.2SαC) and C17.2SαKO cells could also help.

In vivo consequence of TET3/TR interaction

Even though we presented clearly that there is a direct interaction between TET3 and TR, and TET3 was shown to modulate the transcriptional activity of TR, we did not address *in vivo* the functional relevance of this interaction. The main obstacle for *in vivo* study is the insufficient quality of antibodies detecting the endogenous proteins in somatic cells. We dedicated some time and efforts in finding a suitable cell model or tissue with high abundance of both TR and TET3, and we ended up considering brain/nervous system as a good model to study the physiological relevance of TET3/TR interaction.

On one hand, thyroid hormone is known to play important role in fetal and adult brain, it controls a number of developmental and physiological processes in the brain by directly acting on gene expression (Chatonnet F et al, 2015). On the other hand, TET proteins and 5hmC (their catalyzing product) are highly present in brain/nervous system (Globisch D et al, 2010; Munzel M et al, 2010; Szwagierczak A et al, 2010). Recent studies have highlighted their critical role in regulating both neural differentiation and brain function. Unlike in ES cells in which TET1 is most transcribed, TET3 (followed by TET2) is the predominant TET in the brain (Szwagierczak A et al, 2010). TET3 has been reported to be important in mediating early eye and neural development in Xenopus by directly regulating the expression of some key developmental genes (Xu Y et al, 2012). In addition, TET3-null ES cells could be efficiently induced to NPCs (neural progenitor cells), whereas the NPCs undergo apoptosis rapidly and the terminal differentiation of neurons is significantly impaired (Li T et al, 2015). Furthermore, TET3 knocking-down resulted in a significant impairment in fear extinction memory (Li X et al, 2014). Given the high abundance of both TR and TET3 in the brain, an effort in detecting the endogenous interaction is worth trying. Monitoring the correlation of protein levels of TR and TET3 in different brain area and/or at different developmental stages may give an idea of the stabilization effect *in vivo*. Additionally, examining TR protein level change in TET3 knock-out mice would help directly addressing this issue. Moreover, according to the transcriptome studies (Chatonnet F et al, 2012; Royland JE et al, 2008; Diez D et al, 2008), TR possesses very different repertoires of target genes in different brain areas, and at different developmental stages. Whether this cell type-/developmental stage-dependent sensitivity to T3 treatment is correlated with differential TET3 presence would also be worth addressing in future studies.

General events between TET proteins and Nuclear Receptors?

Apart from the two nuclear receptors we discussed above, we observed a similar interaction pattern between TET3 and other members of NR, including AR and ERR, implying that TET proteins may function as a general coregulator for NR. On the other hand, though TET3, among the three TETs, has the

strongest interaction with TR/RAR, the other two TETs have detectable interactions. Given the high homology of the three TET proteins, it is interesting to examine whether the other two TETs have similar modulation effects on TR and/or RAR activity. In this respect, the usage of TETs triple knock-out ES (TKO ES) cells to study the biological relevance of TET3/RAR interaction still worth discussing. The reason we chose ES cell lines is mainly due to the presence of both TET3 and RAR in differentiating ES cells, and the fact that both proteins are involved in modulating differentiation of ES cells. TET1 and TET2 show high expression in ES cells, and while TET1 is gradually down-regulated, TET2 and TET3 are significantly up-regulated during differentiation into the three germ layers (Dawlaty et al, 2013). It should be noted that the relative importance of TET3 among the three TETs at a given stage of ES differentiation remains elusive, as there are no systematically evaluation of absolute protein levels during differentiation of ES cells. Thus studying the biological relevance of three TETs and RAR in TETs triple knock out ES (TKO ES) cells is a compromised choice. The good point of choosing TKO ES is that it enables us to circumvent the redundant role, if any, of three TETs. However, the drawbacks is also obvious that whether the three TETs share similar modulation effect in regulating RAR activity is still a topic remains further investigations. Studying RA response in TET1/2/3 single knock out ES cells, or by reintroducing each TET protein into the TKO ES cells could help addressing this issue.

Cross-talking of TET proteins and NR in cancer?

By virtue of their abilities to regulate a myriad of human developmental and physiological functions, nuclear receptors have been implicated in a wide range of diseases including cancer. For example, androgen receptor (AR) is a critical effector of prostate cancer development and progression (Tan MH et al, 2015); mutations in ER have been shown to be associated with physiological disorders and the manifestation and progression of breast cancer (Saha Roy S et al, 2012). Coregulatory proteins play critical roles in fine-tuning the transcriptional function of nuclear receptors, and studies indicate that altered expression of coregulatory proteins in cancer could modulate tumor cell responsiveness to endocrine therapy, exemplified by SRC3 and NCoR in breast cancer. SRC3 is amplified and overexpressed in a subgroup of primary breast tumors (Anzick SL wt al, 1997), and the elevated SRC3 expression was shown predictive of tamoxifen resistance (Osborne CK et al, 2003). In addition, an evaluation of 160 cases of invasive breast cancer demonstrated that patients with high expression level of NCoR have a better prognosis than those that having low NCoR mRNA expression (Zhang Z et al, 2006). On the other hand, intensive studies relating to TET proteins in cancer revealed that that all three TET genes are mutated in solid tumors, with a high frequency of loss-of function mutation of TET2 in hematological malignancies (Nakajima H et al, 2014). In addition, a downregulation of TET gene expression, which is always associated with decreased 5hmC levels, has been observed in numerous solid cancers, including breast cancer, liver cancer, prostate cancer and lung cancer (Yang H et al, 2012; Hsu CH et al, 2012; Jin SG et al, 2011; Kudo Y et al, 2012). However, the precise impact of altered TET expression/activity on the transformation, progression, and maintenance of these tumors is largely unknown and remains a topic of active research. Functioning as coregulators for NR, whether their dysregulation leads to disruption of NR activity in cancer (such as TETs/AR in prostate cancer, TETs/ER in breast cancer) might open new avenue of research. Identifying link between TET proteins, in conjugation with NR modulation, and

cancer awaits further systematically investigations, this link would open a new avenue for diagnosis and prognosis of cancers and also for identifying potential therapeutic tools to treat cancer.

To conclude, my thesis work identified a new mode of action for TET3, serving as a cofactor of nuclear receptors (specifically TR and RAR). The modulation effects of TET3 on TR and RAR have both similarities and differences. Subjects relating to 1) the exploration of the contribution of the enzyme activity of TET3; 2) the biological relevance of TET3/TR interaction; 3) the generalization the study to TET proteins and nuclear receptors; 4) the observation of the cross-talking between TETs and NR in cancer; awaits further studies.

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