Chapter 9
Integrative Genomics to Dissect Retinoid Functions

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Abstract Retinoids and rexinoids, as all other ligands of the nuclear receptor (NR) family, act as ligand-regulated *trans*-acting transcription factors that bind to *cis*-acting DNA regulatory elements in the promoter regions of target genes (for reviews see [12, 22, 23, 26, 36]). Ligand binding modulates the communication functions of the receptor with the intracellular environment, which essentially entails receptor-protein and receptor-DNA or receptor-chromatin interactions. In this communication network, the receptor simultaneously serves as both intracellular sensor and regulator of cell/organ functions. Receptors are "*intelligent*" mediators of the information encoded in the chemical structure of a nuclear receptor ligand, as they interpret this information in the context of cellular identity and cell-physiological status and convert it into a dynamic chain of receptor-protein and receptor-DNA interactions. To process input and output information, they are composed of a modular structure with several domains that have evolved to exert particular molecular recognition functions. As detailed in other chapters in this volume, the main functional domains are the DNA-binding (DBD) and ligand-binding (LBD) [5–7, 38, 56, 71]. The LBD serves as a dual input-output information processor. Inputs, such as ligand binding or receptor phosphorylations, induce allosteric changes in receptor surfaces that serve as docking sites for outputs, such as subunits of transcription and epigenetic machineries or enzyme complexes. The complexity of input and output signals and their interdependencies is far from being understood.

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Abbreviations

9-*cis* RA	9-*cis* retinoid acid
AM580	RARα-selective synthetic ligand
apoNR	Non-liganded NR
at-RA	All-*trans* retinoic acid
BMS753	RARα-selective synthetic ligand
BMS961	RARγ-selective synthetic ligand
CD437	RARγ-selective synthetic ligand
Cistrome	The total set of genes in a given cell that contains *cis*-acting DNA binding/response/target sites for a given TF; generally defined by ChIP-seq and related technologies
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP coupled to massive parallel sequencing
CoA	Co-activator
CoR	Co-repressor
DBD	DNA-binding domain
ECC	Embryo carcinoma cell (e.g., F9 or P19)
ESC	Embryonic stem cells
Epigenome	General term to describe the patterns of post-translational modification of chromatin histones alone the genome and the modification of DNA, such as methylation or hydroxymethylation of cytosines
HDAC	Histone deacetylase
IP	Immunoprecipitation
IPed	Immunoprecipitated
Isotype	Three RAR and RXR receptors expressed from distinct genes (RARα, RARβ RARγ; RXRα, RXRβ and RXRγ)
LBD	Ligand-binding domain
MEF	Mouse embryonic fibroblast
NR	Nuclear receptor
RAR α, β, γ	Retinoic acid receptor α, β, γ
RXR α, β, γ	Retinoid X receptor α, β, γ
TF	Transcription factor
Transcriptome	All transcribed RNAs produced in one or a population of cells.

Retinoid receptors, RARs and RXRs, are each expressed from the three isotypic genes (α, β and γ), which express isoforms by differential promoter usage and splicing [36]. RAR and RXR isotypes form heterodimers, and RAR isotype-selective and RXR-selective ligands have been developed [12]. While RAR-RXR heterodimers respond to cognate RAR ligands, RXR ligand action requires prior RAR ligand binding (termed RXR '*subordination*', a phenomenon that is molecularly well understood [24] but does not apply to all RXR heterodimers.

The early steps of nuclear receptor function and the physiological impact of retinoic acid receptor (RAR) heterodimers are rather well understood. Numerous molecular, structural, and structure-activity relationship studies have informed us

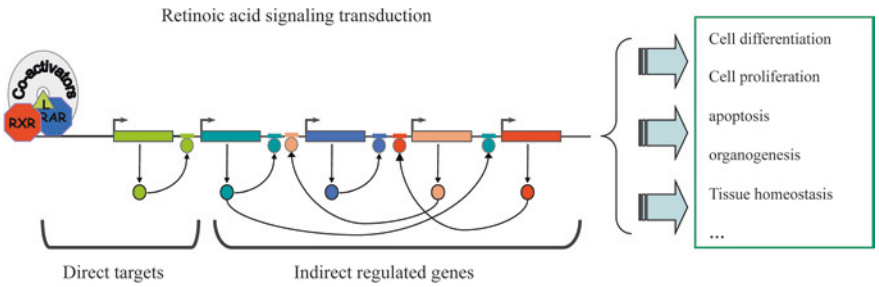

Fig. 9.1 Schematic representation of the retinoic acid signaling transduction process

about the sequence of events that follows ligand binding, and we understand how these events can be modulated by ligand design [9, 26, 53]. However, how a single ligand, such as retinoic acid (RA), that binds to multiple receptors regulates a plethora of cell-specific dynamic networks of genes and how the epigenome contributes to transcriptional regulation that ultimately reads out as a cell-level, physiological phenomenon, is still a black box (Fig. 9.1). It is our view that it is time to develop a *systems biology of nuclear receptor action*. Due to advances in massive parallel sequencing and bioinformatics analyses of genome-wide data sets, such a quest is possible. Indeed, it is now possible to integrate data on global transcription factor binding, epigenetic chromatin histone and DNA modification patterns with transcriptome and 3-dimensional chromatin structure data. *Decision* points that govern temporal control points in gene networks that are the ultimate genetic readouts of the RA- (or, more generally, NR-) induced physiological phenomena can be extracted and deciphered from these integrations. Here, we will discuss the chronology of the development of increasingly larger data sets for RA action and provide an overview of present attempts to integrate a multitude of genome wide data sets in the context of a 4-dimensional appreciation of chromatin structure and activity.

History: Retinoic Acid Signaling in the Post-Genomic Era

With the publication of the first draft of the human genome sequence in 2001 and subsequently, various other model organisms, the molecular genetics behind organismal homeostasis has entered into a new era. In fact, studies on the regulation of biological phenomena are now generally performed in the context of available genome sequences and high throughput technologies for diverse applications, such as the expression of all nascent or specific classes of RNAs, the global binding of a transcription factor ('cistrome'), the genome-wide post-translational modification of chromatin ('epigenome') or the 4-dimensional organization of chromatin in space and time.

Importantly, this new way of interrogating the genome generates a greater number of significant targets than those identified in previous years by standard

genetics/molecular biology approaches, and is thus, expected to provide a more comprehensive view of the regulatory events during RA-signaling. In fact, a review published in 2002 summarized the efforts of more than 1,191 published articles that classified 532 genes as regulated targets of the RA-signaling pathway [4]. In that same year, Geoffrey Childs and colleagues, studying the genetic basis for RA-induced differentiation of F9 embryo carcinoma cells (ECCs) into parietal endoderm and the RA-induced differentiation of P19 ECCs into neurons, characterized more than 500 differentially regulated genes ([29, 70]; Table 9.1). This increased discovery rate was made possible by an early version of microarray technology (cDNA PCR-spotted microarrays; reviewed in [40]. Interestingly, the two studies assessed differential gene expression at different time-points during RA-induced cell fate transitions. The corresponding functional genome annotation and temporal gene expression patterns offered a first insight into the signal transduction pathways involved in endodermal and neuronal differentiation.

To shed light on whether the expression of the genes was directly dependent on or regulated by the presence of a liganded and DNA-bound, RAR/RXR heterodimer, Harris and Childs [29] selected immediate response genes by inducing F9 differentiation with all-*trans*-RA (*at*-RA) for 6 h in the presence of the protein biosynthesis inhibitor, cycoheximide. Under these conditions, they identified 109 genes that displayed significant differential induction. Of these, only 22 were validated in a 9 day *at*-RA-exposure, time-course assay, suggesting that the other genes were false-positives due to the cycloheximide treatment.

Subsequent global transcriptomics studies were carried out using different model systems to try to discriminate putative primary/direct and secondary/indirect RA-responsive genes by systematically querying early and late treatment time points [16]. Others took advantage of the RAR-specificity of the *at*-RA synthetic analog, TTNPB, as a way to further increase the specificity of the assay [2, 42]. Although the underlying rationale was that the pan-RAR agonist, TTNPB, would more specifically identify RAR-responsive genes by decreasing the potential to inadvertently identify genes that were responding to permissive *9-cis*-RA-bound RAR/RXR heterodimers rather than *at*-RA-bound receptor complexes, the use of TTNPB does obviously not discriminate between the contributions of the different RAR/RXR isotype heterodimers.

Using RAR isotype-selective knock-out F9 ECC cells, Lorraine Gudas and her colleagues studied the role of RARγ by performing global gene expression profiling with wild-type and RARγ-/- cells in the presence or absence of *at*-RA [63]. Earlier studies [10, 11, 65] had demonstrated that the use of a RARγ specific ligand, and not those targeting the RARα or RARβ isotypes, drives *at*-RA induced F9 cell differentiation. The global gene expression study by Su and Gudas demonstrated that wild type and mutated RARγ$^{-/-}$ cells presented with similar proliferation and morphological characteristics in the absence of RA treatment, but displayed important differences in their gene expression profiles. A similar observation was made with mutated RARα$^{-/-}$ F9 cells [37], suggesting that RARα and RARγ possess *ligand-independent* gene regulatory functions. Notably, these studies, in concordance with earlier studies [10, 11], reveal there are serious limitations in using RAR knockout cell lines to decipher the specific roles of RAR

Table 9.1 Published studies focused on dissecting retinoid function by applying "omics" approaches

Publication source	Relevant feature	Omics methodology
Harris and Childs [29]	Global gene expression of RA/dibutyryl cAMP-induced F9 parietal endoderm differentiation (kinetics over 9 days of treatment)	PCR-spotted microarrays (8,900 mouse cDNAs)
Wei et al. [70]	Global gene expression of RA-induced P19 neural differentiation (kinetics over 8 days of treatment)	PCR-spotted microarrays (9,000 mouse cDNAs)
Arima et al. [2]	Global analysis of RAR-responsive genes in the Xenopus embryo treated with the RAR-specific ligand TTNPB	Xenopus EST microarray (EST clones from NIBB Mochii normalized Xenopus neurula library (19,200 clones) and tail bud library (23,040 clones)
Eifert et al. [16]	Global gene expression of RA-induced F9 primitive endoderm differentiation. Two time-points (8 and 24 h under ATRA treatment) were evaluated relative to the absence of treatment condition	Atlas mouse 1.2 cDNA expression array (BD biosciences clontech; 1,176 mouse cDNAs plus 9 housekeeping cDNAs in a nylon membrane format); Affymetrix murine genome U74Av2 gene ChIP oligonucleotide microarrays (12,488 unique genes per array)
Mamoon et al. [42]	RA-responsive genes assessed in murine hepatocyte cell line (AML12) treated with ATRA as well as with the RAR-specific ligand TTNPB	Affymetrix Genechip mouse genome 430 2.0 microarrays (39,000 transcripts represented)
Su and Gudas [63]	Global gene expression in wild type and RARγ-/- F9 cells with and without RA treatment	Affymetrix Genechip® arrays
Hua et al. [30]	Global gene expression and e-GFP tagged RARα/RARγ binding sites assessed in MCF7 breast cancer cells under RA treatment	Agilent human genome oligo microarrays (gene expression); Affymetrix GeneChIP® Human tiling 2.0R arrays (ChIP-chip)
Delacroix et al. [13]	Overexpressed RARα and RARγ chromatin localization in MEF and ES cells under ATRA treatment	Agilent promoter arrays (ChIP-chip)
Mahony et al. [41]	Global gene expression and pan-RAR localization in mES cells before and after RA-treatment	Affymetrix mouse genome 430 2.0 microarrays (gene expression); Solexa sequencing (ChIP-seq)
Delacroix et al. [48]	Global gene expression in F9 cells under ATRA and RAR specific agonists. RXRα and RARγ binding sites assessed under ATRA treatment (kinetics over 48 h)	Affymetrix mouse GeneChIP® microarrays (gene expression); Solexa sequencing (ChIP-seq)

isotypes. This is because there can be artifactual ligand responses by RAR/RXR heterodimers in RAR isotype knockouts. This suggests that the normal role(s) of each RAR isotype, as deduced from studies of RAR-RXR knockout cell models, requires cautious interpretation.

Genome-Wide Mapping of Retinoic Acid Receptors Binding Sites

Dissection of the effects of RA on physiological processes requires a comprehensive mapping of the chromatin interaction of the various RAR/RXR heterodimers before and after ligand exposure (Fig. 9.1). Previous in vitro binding and transactivation studies demonstrated that RAR/RXR heterodimers bind efficiently to inverted (IR) or direct repeat (DR) sequences of the hexameric motif (A/G)G(G/T)TCA, often spaced by 5, 2 or 1 nucleotides (DR5, DR2, DR1) due to the dimerization characteristics of the DNA binding domain [54, 67, 74, 75]. While this characteristic RA Response Element (RARE) could in principle allow the identification of all potential RAR/RXR binding sites in a given genome, the sequence motifs of some RAREs associated with well-known RA-induced genes demonstrated major divergence from the consensus motif [35]. Available evidence indicates that although consensus RAREs may be efficient and correspond to high affinity binding sites, they rarely occur in natural RA target genes. In addition, relying on consensus sequences to identify RA-regulated genes does not take into consideration additional epigenetic mechanisms that regulate access of RAR/RXR heterodimers and transcription factors to chromatin [33, 34], the action of "*pioneer transcription factors*" [73], or synergistic interactions with or tethering to other NR/TFs [57].

At present, the methods of choice for comprehensive and unbiased mapping of protein-chromatin interactions are a combination of chromatin immunoprecipitation (ChIP) with high throughput profiling approaches like ChIP-chip and more recently, ChIP-seq (Fig. 9.2). DNA biochips, also called DNA arrays, were first described in 1995. This technology provides a method for interrogating *protein-DNA-chromatin* associations by first, co-immunoprecipitating protein-DNA fragments, and then hybridizing the captured DNA on a solid support coated with an arrangement of single-stranded DNA molecules (commonly referred to as probes) covering, for instance, the complete genome sequence of a selected species. The assay resolution of this approach, commonly called *ChIP-chip* (Fig. 9.2) is directly related to the number of genomic probes that can be spotted onto the solid support and its sensitivity depends on the minimal amount of co-immunoprecipitated DNA fragments required per assay. ChIP-chip was widely used until the arrival of second generation genome sequencers that were able to provide faster, less expensive, and more direct ways to evaluate the diversity of co-immunoprecipitated DNA fragments. Both assay resolution and sensitivity were highly increased using *ChIP-seq* technology because it involves direct sequencing of the immunoprecipitated fragments (Fig. 9.2), thus accounting for the current overwhelming use of this approach.

Among the first studies to apply global approaches for mapping chromatin localization of RARs were those carried out by Delacroix and colleagues [13] using mouse embryo fibroblasts (MEFs) and by Hua and colleagues [30] using human MCF-7 breast cancer cells. The MEF study attempted to discriminate between direct and indirect RA-regulated targets using Taf4$^{lox/-}$ MEFs, which undergo morphological changes upon RA treatment accompanied by changes in the expression of more than 1,000 genes [19]. After integration of 3xFlag-HA tagged RARα or RARγ isotypes, which allowed immunoprecipitation with anti-Flag and anti-HA antibodies, they performed ChIP assays and hybridized the immunoprecipitated DNA to Agilent arrays coated with DNA encoding the promoter regions of around 17,000 genes (ChIP-chip) [13]. They identified ~300 RAR-occupied sites of which less than 25 % corresponded to differentially expressed RA target genes. In part, the low correlation between RAR occupancy and actual functional relevance can be explained by the design of the assay; that is, ChIP-chip was performed with MEFs treated for 2 h with RA, while the transcription profiling was done with MEFs treated for 24 h. This highlights the potential risks in comparing different sources of global information and the need to rigorously design assays with appropriate attention to matched conditions of targets and probes and normalization of datasets.

Prior to the MEF study, using a conceptually similar approach, eGFP-tagged RARα or RARγ were integrated in human MCF-7 breast cancer cells to allow characterization of the role of specific RAR isotypes in mediating the anti-proliferative and apoptotic effects of RA [30]. In this case, immunoprecipitated chromatin was hybridized to tiling arrays containing more than 40 million oligonucleotide probes that represented the entire human genome. More than 3,000 RARγ and more than 7,000 RARα binding sites were found under these conditions. Importantly, more than 85 % of the identified sites were located in intronic or promoter-distal intergenic regions. In addition, the transcriptional response in the MCF-7 model system was evaluated with *at*-RA, as well as with the RARα-specific agonist, AM580 and the RARγ specific-agonist, CD437. Moreover, the authors used RARα and RARγ isotype-specific RNA interference to link the differential gene expression seen with isotype-selective ligands with presence of the corresponding RAR isotype, and found a high degree of correlation. This study also demonstrated an unexpected competition between RAR/RXR heterodimers and the estrogen receptor ERα for binding sites, suggesting there may be antagonistic transcription regulation for up to 71 % of the evaluated target genes.

Development of the Field: Identification of Decision Points and Key Factors that Diversify and Dynamically Regulate RA-Induced Gene Expression

More recently, a similar study was carried out with mouse embryonic stem cells (ESC) to identify the RA-dependent gene programs involved in neuronal differentiation [41]. A pan-RAR antibody was used to map endogenous RAR chromatin

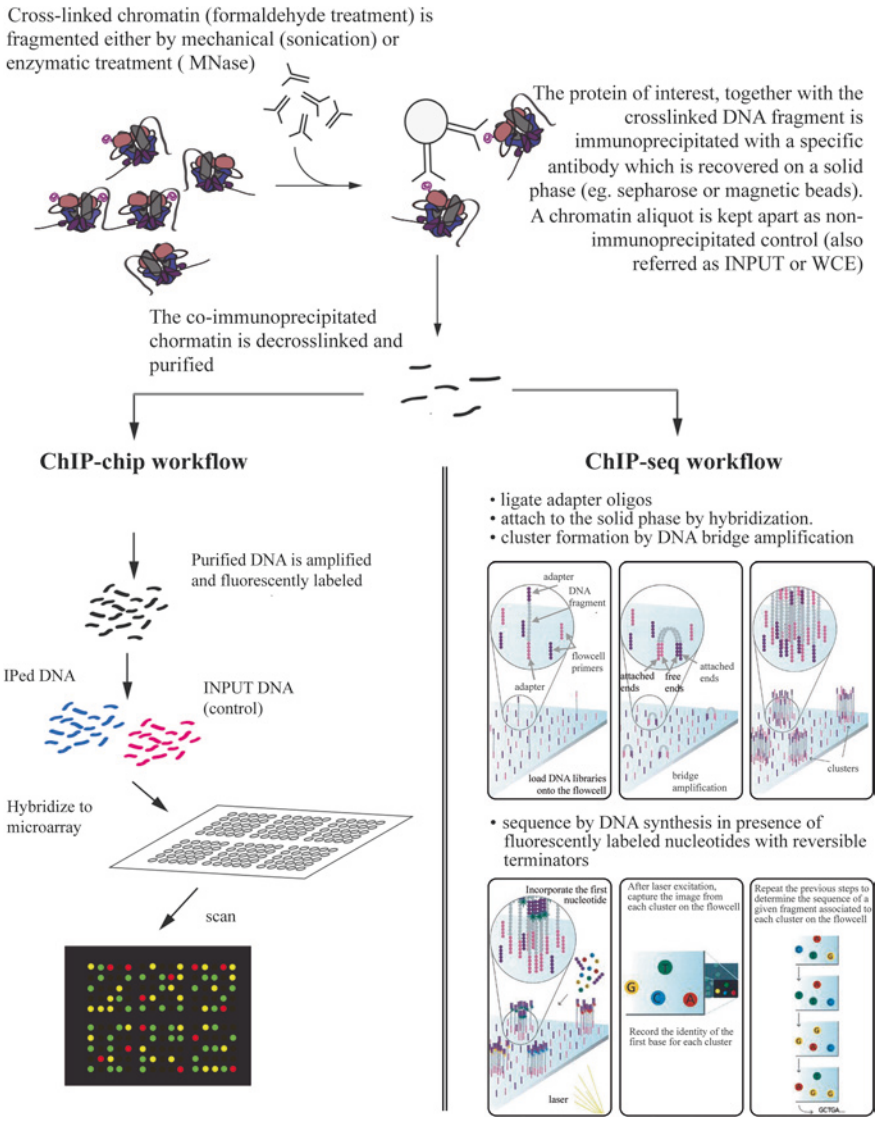
Cross-linked chromatin (formaldehyde treatment) is fragmented either by mechanical (sonication) or enzymatic treatment (MNase)
The protein of interest, together with the crosslinked DNA fragment is immunoprecipitated with a specific antibody which is recovered on a solid phase (eg. sepharose or magnetic beads). A chromatin aliquot is kept apart as non-immunoprecipitated control (also referred as INPUT or WCE)
The co-immunoprecipitated chormatin is decrosslinked and purified
ChIP-chip workflow
Purified DNA is amplified and fluorescently labeled
IPed DNA
INPUT DNA (control)
Hybridize to microarray
scan
ChIP-seq workflow
• ligate adapter oligos
• attach to the solid phase by hybridization.
• cluster formation by DNA bridge amplification
adapter
DNA fragment
flowcell primers
adapter
load DNA libraries onto the flowcell
attached ends
free ends
attached ends
bridge amplification
clusters
• sequence by DNA synthesis in presence of fluorescently labeled nucleotides with reversible terminators
Incorporate the first nucleotide
laser
After laser excitation, capture the image from each cluster on the flowcell
Record the identity of the first base for each cluster
Repeat the previous steps to determine the sequence of a given fragment associated to each cluster on the flowcell
GCTGA...

Fig. 9.2 Schematic comparison between chromatin immunoprecipitation assays evaluated by hybridization onto DNA biochips (ChIP-chip) and massive parallel sequencing (ChIP-seq). In ChIP-chip assays, the immunoprecipitated DNA is hybridized with a solid surface (biochip) previously coated with single-stranded DNA molecules (referred as probes) representing for instance, a complete genome. In the illustrated example (*left panel*), the immunoprecipitated DNA and the non-immunoprecipitated control (also referred to as INPUT or WCE for whole cell extract) are labeled with two different fluorophores and then hybridized together on a single DNA biochip. An alternative approach is based on hybridization on two different DNA biochips, followed by a computational comparison of the imaged fluorescent levels. ChIP-seq assays are based on the direct sequencing of the immunoprecipitated DNA by using a massive parallel sequencing approach. Briefly it consists of the incorporation of adapter sequences at both ends of the immunoprecipitated DNA; then such adapters are used for attaching the DNA molecules onto a solid surface coated with single-strand DNA molecules representing the complementary sequence to the adapters in use. The attached molecules are amplified by following several rounds of "bridge DNA amplification" based on the alternate attachment of the adapters in use to the solid surface (*right panel*). Bridge amplification produces DNA clusters formation, which are then sequenced by DNA synthesis in the presence of fluorescently labeled nucleotides with reversible terminators. This illustrated procedure corresponds to that developed by the company Solexa. Other massive parallel sequencing approaches have also been developed for this purpose

binding sites, thus avoiding the potential for over-expressed or tagged constructs to identify artifactual binding sites. The assay, performed on cells exposed to *at*-RA for 8 h, revealed both constitutive and RA-induced, de novo binding sites. ChIP-seq studies were performed to further assess the role of the RAR binding sites on transcription regulation, global microarray-based gene expression, and RNA polymerase II initiation and elongation. The number of genes differentially regulated by RA was estimated using a 5 kb proximity criterion that predicted a link between RAR binding sites and *proximal* transcriptionally active, coding regions. Clearly, RA-responsive genes can also, in principle, be regulated by *distal* enhancers, which cannot be identified by such a simplified binding site proximity criterion.

Our own recent study used the well-established F9 embryonal carcinoma cell line (ECC) model to dissect the gene regulatory pathways responsible for RA-induced endodermal differentiation. This was done by integrating global RAR binding and gene regulation information from samples collected at five different time-points over the course of 48 h exposure of the cells to *at*-RA or RARα, β, or γ-specific agonists [48].

In contrast with results obtained using MCF7 cells [30], F9 cells treated with an RARγ-specific agonist, BMS961 (but not those exposed to an RARα-specific agonist, BMS753), induced a pattern of differential gene expression that was similar to that induced by *at*RA [48]. This is consistent with previous studies demonstrating that the RARγ agonist (but not those of RARα or RARβ) induces an F9 cell differentiation phenotype that is indistinguishable from the one induced by the natural ligand, *at*-RA, suggesting that RARγ is driving differentiation in F9 cells [65].

Given the decisive role of RARγ in F9 cell differentiation, we identified the chromatin binding sites of RARγ/RXRα heterodimers by mapping each receptor separately at all 5 time-points. Overall, RXRα displayed more binding sites than RARγ, as was expected from the promiscuous heterodimerization of RXRα with

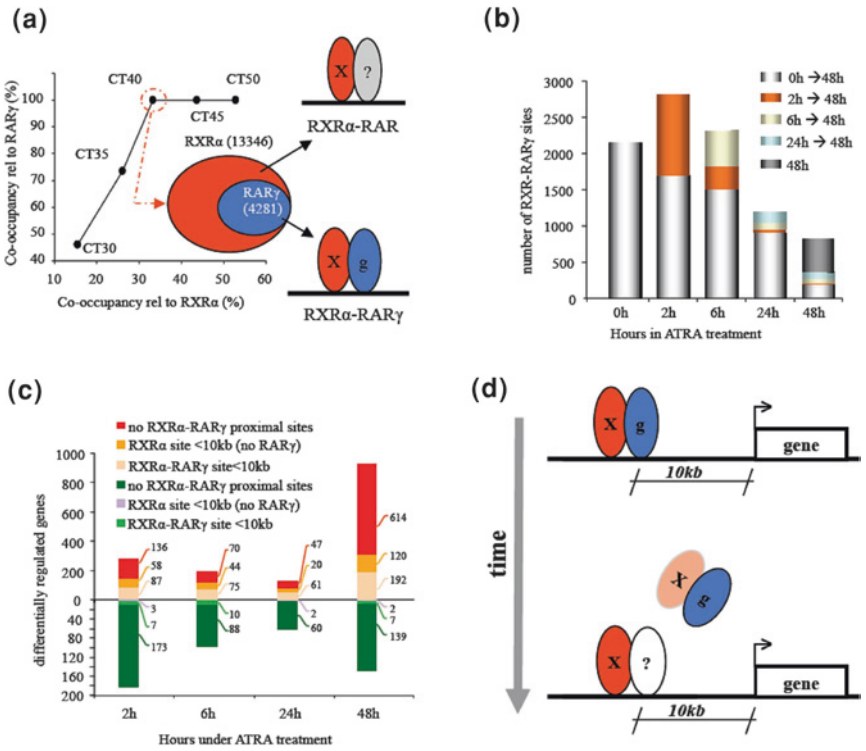

Fig. 9.3 RXRα and RARγ nuclear receptors present a highly dynamic binding to chromatin during ATRA-induced F9 differentiation. **a** The percent of RXRα and RARγ co-occupancy relative to the total number of RXRα or RARγ binding sites retrieved over all time-series evaluated profiles is illustrated for different P-value confidence thresholds (CT = −10*log (P-value)). The inset (Venn diagram) shows that at CT = 40 all identified RARγ sites are found co-occupied with RXRα. This subset of binding sites is considered bona fide RXRα/RARγ heterodimer binding sites and has been used for all further analysis. **b** The RXRα/RARγ binding sites identified in (**a**) are illustrated in the context of their temporal recruitment, duration of occupancy and dissociation. RXRα/RARγ co-occupied sites per time point are subclassified based on their recruitment intervals and depicted by colour coding. **c** Genes exhibiting ATRA-induced or repressed mRNA levels at the indicated time points during F9 cell differentiation (induced genes ≥1.8-fold; repressed genes ≤0.5-fold relative to vehicle) were classified as putative target genes if at least one RXRα or RXRα/RARγ binding site was located in proximity (≤10 kb distance). **d** Schematic model illustrating the progressive loss of RARγ but not of RXRα from chromatin binding sites observed during ATRA-induced F9 differentiation

multiple partners (Fig. 9.3a). Merging the datasets and extracting binding sites that were common to both RXRα and RARγ revealed a population of constitutive RARγ/RXRα binding sites plus a population that was highly dynamic during *at*-RA treatment. The overall number of RARγ/RXRα binding sites decreased during F9 differentiation (~2,000 sites in the absence of treatment and less than 1,000 sites after 48 h exposure of cells to *at*-RA), and we detected significant amounts of de novo recruited heterodimers even after 24 h of *at*-RA treatment (Fig. 9.3b, c). These results suggested a sustained and highly dynamic interaction of the

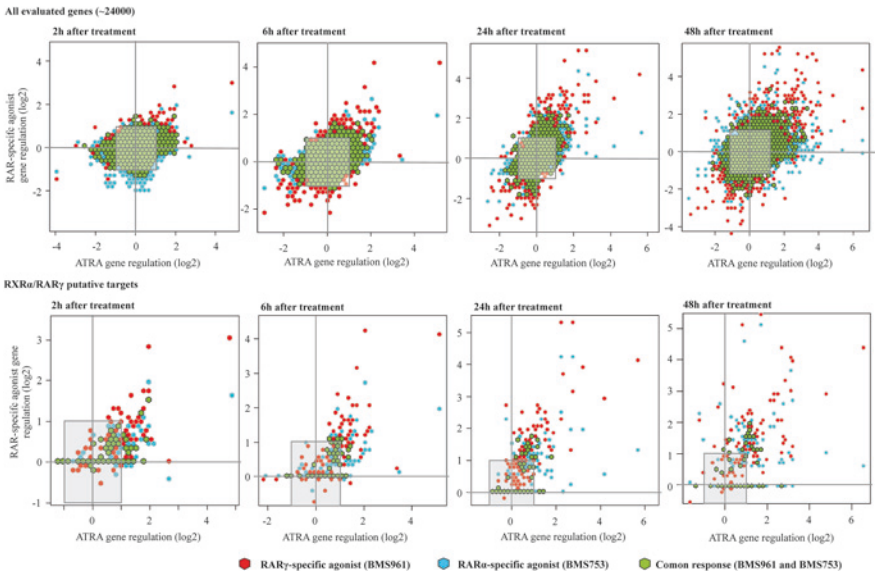

Fig. 9.4 Differential gene expression response induced by ATRA treatment in comparison to that induced by the RAR-specific agonists. The *upper panels* illustrates the gene expression response induced in all evaluated coding regions (24,000 genes; Affymetrix mouse GeneChIP® microarrays), while the *lower panels* displays the response in the characterized RXRα/RARγ putative target genes. Gene regulation response induced either by the RARγ-specific agonist BMS961 (*red*), by the RARα-specific agonist BMS753 (*light blue*) or by both ligands (*green*) are displayed in the context of the ATRA-induced response. The *central box* in each panel delineates a gene expression response area lower than 2 folds, thus the significant gene regulation responses are found outside of this delimited surface

RARγ/RXRα heterodimer with chromatin targets during this cell physiological process. The observed decrease in binding sites of RARγ/RXRα heterodimers relative to the total number of RXRα-occupied sites may result from an exchange of RXRα with other NR partners during the differentiation (Fig. 9.3d).

We found that more than 50 % of the genes induced during the first 24 h of *at*-RA treatment showed a RXRα or an RARγ/RXRα binding site within 10 kb proximity (Fig. 9.3c). In contrast, most of the down-regulated genes lacked such sites. Importantly, more than 70 % of the mapped RXRα sites could not be associated to an annotated coding region, suggesting that they might regulate transcription through 3-dimensional chromatin structures or may regulate as yet non-annotated transcripts. To further confirm direct transcriptional regulation by RARγ/RXRα binding sites, we compared transcriptional responses in cells exposed to *at*-RA or RAR-specific agonists (Fig. 9.4) [48]. Approximately 60 % of the *at*-RA-induced putative RARγ/RXRα targets did respond similarly to the differentiation competent RARγ agonist BMS961. Surprisingly, however, ~40 % responded also to the RARα agonist BMS753. This suggests that (1) ~40 % of the *at*-RA-induced putative RARγ/RXRα targets that did not respond to the BMS961 treatment require, or can operate in a redundant manner with other RAR isotypes,

are not essential for differentiation, and (2) another 40 % of the *at*-RA-induced putative RARγ/RXRα targets that responded to both BMS961 and BMS753 display a promiscuous response to both RARα and RARγ, which alone is not sufficient, but may possibly support development of the differentiation phenotype.

Overall, the integrative analysis combining the dynamic regulation of gene expression by receptor-selective ligands with the chromatin binding of the corresponding heterodimers largely facilitates identification of direct RAR isotype and heterodimer-selective regulated target genes.

Current State of the Field: Taking Advantage of in Silico Integrative Approaches to Expand Understanding of RA-Driven Signal Transduction Processes

The integrative analysis of global gene expression and RXR and RAR chromatin association can, in principle, identify an important proportion of RAR/RXR heterodimer-mediated gene regulatory events. Including a temporal dimension revealed a highly dynamic target gene expression profile and dynamic occupancy of chromatin by pre-existing and de novo recruited RAR/RXR heterodimers, as well as heterodimer replacement or even partner swaps.

The gain of information remains restricted to directly regulated RAR/RXR heterodimer-targets, which represent only a small fraction of all differentially RA-regulated genes. The remaining majority of regulated genes are generally referred to as indirectly or secondarily regulated genes. It is reasonable to assume a hierarchical order of transcription regulation in which the direct targets are in the front line of the signaling process (‘*initiator program*’) and the downstream layers comprise temporally specified (‘*executor*’) gene programs that result in amplification, diversification and specification of secondary gene programs that determine cell fate and ultimately, cell differentiation. The initiation phase is mediated mainly, albeit not exclusively, by TFs. Our studies have confirmed that many TFs are among the early genes. Therefore, the reconstruction of the executor programs may profit from the characterization of the cascade of TFs that propagate the signal transduction and diversification process. In past years, the chromatin location of various TFs has been mapped in several model systems by ChIP-chip or ChIP-seq approaches and released to public repositories, thus generating an important resource for in silico omics dataset integration. Importantly, the integration of TF target gene information into time course gene expression data is a powerful method for identifying downstream regulatory events during a signal transduction process [18]. To deconvolute RA signaling pathways during F9 induced differentiation, we have integrated TF target gene annotation, including identified direct putative RARγ/RXRα targets, into the *at*-RA–induced gene programming [48]. For this we used the recently developed Dynamic Regulatory Events Miner (DREM; [18], which uses input data for temporal alteration of gene expression at given time points and transcription factor-target gene interactions. The underlying

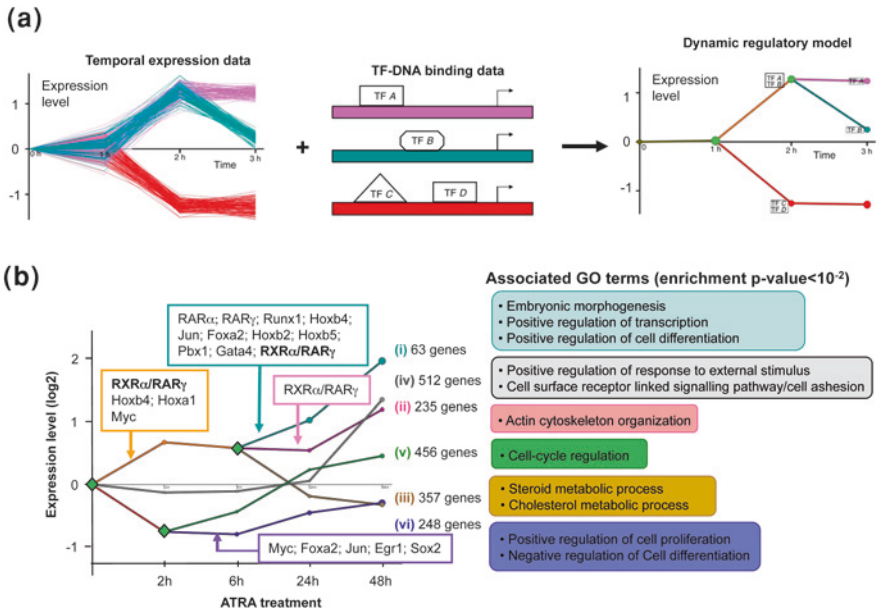

Fig. 9.5 Reconstructing a dynamic regulatory map for the RA-driven transcriptome. **a** Shematic representation of the integrative approach used by DREM. Temporal gene expression information (*left panel*) is combined with transcription factors-DNA binding annotations (*middle panel*) to infer a dynamic regulatory model. In the illustrated example, co-expressed genes are classified in three major paths (coloured in *pink*, *green* and *red* respectively) which in addition can be associated to a defined TFs based on the TF-DNA binding annotations (i.e. *pink* genes are regulated by TF A, *green* genes by TF B and *red* genes by TF C and TF D). In this manner, DREM aims at assessing how likely is that a given group of co-expressed genes may be transcriptionally regulated by a given TF. **b** DREM co-expression analysis is represented by *colour*-coded paths that summarize common characteristics. *Diamonds* indicate the predicted bifurcation points giving rise to the different co-expression paths and transcription factors whose target genes are overenriched in a given path are also illustrated. The number of genes per co-expression path, as well as their relevant gene ontology terms is displayed in aside. Panel **a** has been adapted from Ernst [18]

hypothesis is that various co-expression events (described here as co-expression paths) derive from defined transcriptional regulatory decisions. Integrating the information retrieved from TF target gene annotations from time course analyses of gene expression patterns will allow predictions for the involvement of a given TF in the formation of a defined co-expression path (i.e., at bifurcation points, as illustrated in Fig. 9.5). In the case of RA-induced differentiation of F9 ECC, DREM analysis predicted six distinct gene co-expression paths that recapitulate the different subprograms generated during RA-induced signal transduction. DREM allows evaluation of whether a given co-expression path is enriched for genes that are annotated as targets of a specific TF and whose actions contribute to the predicted bifurcation. In our analysis, three bifurcation points leading to signal diversification were identified along with candidate TFs predicted to cause diversification. As proof-of-principle, DREM correctly associated RARγ/RXRα

with upregulated subprograms validated by differential gene expression and the chromatin-binding pattern of RARγ/RXRα (Fig. 9.5). Notably, DREM predicted that homeobox family transcription factors (e.g., Hoxa1, Hoxb2, Hoxb4, Hoxb5) and others like RARα or Foxa2, were enriched in upregulated subprograms, whereas TFs like Egr1 [50] and Sox2 [52] (TFs associated with stem cell renewal rather than differentiation), were associated with the repressed path.

The predicted RA-induced co-expression paths were further evaluated in the context of bibliographic gene co-citation interactions in order to construct predicted RA-driven RARγ/RXRα-mediated signaling networks [48]. This type of analysis correlates relevant genes, like the ones described above with their bibliographic co-citation '*partners*', which helps in assigning functional features to the predicted subprograms. Such an analysis illustrates the complex temporal coordination of the diverse molecular processes involved in RA-induced differentiation and predicts critical nodes are associated with cell fate transitions initiated by RA.

Relevance: Importance of a Systems Biology of Nuclear Receptors

Early studies in *Drososphila* paved the way toward a systems biology view of NR action. These studies characterized the temporal programming of gene activation induced by the steroid hormone, ecdysterone, to initiate molting and metamorphosis [3]. This was possible because gene activation manifests itself as local, reversible, alterations (*puffs*) of the polytene chromosomes that comprise approximately 1,000 chromatids in the interphase nuclei in the salivary glands of 3rd instar larvae. Temporal alteration of the puffing pattern provided a readout of sequential activation of gene programs. Early puffs corresponded to direct activation of TF-encoding, target genes by the ecdysone receptor [59, 66]. The ecdysone receptor turned out to be a heterodimer [72]. These features of the fly gene program share similarity with RAR/RXR-mediated activation of genetic sub-programs [48]. Thus, it is likely that the principles of the temporal gene programming seen for the ecdysone and retinoid receptors correspond to a general mechanism for signal diversification and temporal programming of hierarchical, downstream gene programs.

However, there are several additional factors and regulatory paradigms that impact program execution. One involves the surprising dynamics of RAR/RXR heterodimer binding [48]. These dynamics are characterized by heterodimer binding and dissociation at all time-points during the observation period of 48 h. As well, extensive RXRα partner swapping is observed, with either one RXRα heterodimer dissociating and being replaced by another one, or with a '*partner swap*' occurring while the heterodimer is bound to chromatin by an as yet, unknown, mechanism [48]. The effects of such heterodimer swapping on the dynamics of co-regulator-receptor complexes at target chromatin during cell differentiation has not been addressed with global approaches. This missing piece deserves attention,

particularly in view of potentially distinct preferences of RXR heterodimers for co-regulators that are recruited to resulting multi-protein (epigenetic) complexes. Receptor-co-regulator interaction may correspond to a mechanism of target gene specification, and, vice versa, upon binding, the target DNA may allosterically alter receptor structure and function [43, 76].

Some, if not most, TFs that are regulated during the sequence of events governing cell fate determination have the ability to act as pioneer factors [73]. Such factors *open* chromatin structure thereby generating DNAse I hypersensitive sites that are otherwise not accessible to TFs that lack pioneering activity [69]. Obviously, one salient feature of the pioneering concept is that it provides a basis for the hierarchical and temporal order for the execution of gene programs.

Future Directions: An Integrative Genomics Era

How can the structural information present in a simple chemical molecule like *at*-RA set-up the sequence of temporally controlled events that finally lead to a differentiated cell? The F9 ECC global gene profiling studies have provided for the first time a systems biology view that RA-induced signaling comprises a diverse series of events that set in motion different regulatory decisions which occur in a time-defined manner throughout cell differentiation [44, 48]. Yet this view is far from comprehensive. In part, this is due to technical constraints related to the complexity of a system that operates with up to six receptors and multiple heterodimers. But it is also a consequence of the reduced number of molecular events that can currently be imported into spatio-temporal omics dataset analyses.

Multiple RXR: RAR Heterodimers Mediate RA-Signaling

The first level of signal diversification results from the multiplicity of RAR/RXR complexes that can be formed, which is a function of the expression levels of the six different RAR/RXR isotypes in a given cell (RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ). Our recent study showed that it is possible to dissect the gene program regulated by the RARγ/RXRα heterodimer in a specific manner; however, the contribution of other RAR/RXR heterodimers RXRα partner swapping remain to be elucidated. Exploring the role of other RAR/RXR heterodimers depends on the availability of high quality "ChIP-seq grade" antibodies and ChIP-seq profiles. Note that in this respect there may be significant differences in the quality of ChIP-seq profiles calling for rigorous quality control assessment of data sets to allow integrative data analysis [47]. While this limitation can be overcome by using stably expressing epitope-tagged receptors, the risk of an altered functionality imposed by tagged receptors and potential interference of these modified receptors with

regulation by endogenous receptors cannot be rigorously excluded. Notably, the heterodimer swap we observed between RARγ/RXRα and RARα/RXRα at the RARβ2 promoter is a note of caution for the use of exogenous modified factors.

Although RA signaling is mediated by heterodimers, this does not imply that both partners are located in the same chromatin region at the same time and in the same cell. Therefore, subsequent *reChIP-seq* assays, i.e. sequential ChIPs with antibodies directed against the two partners, followed by massive parallel sequencing, are necessary to provide reliable information about co-occupancy of the evaluated heterodimer partners at a given chromatin site. While reChIP assays have been shown to be a powerful method for evaluating simultaneous co-occupancy events in a locus-centric manner [8, 49], the low yields are not compatible with the requirements for global ChIP-seq assays. To overcome this problem we have recently combined reChIP assays with linear DNA amplification and sequencing (LinDA-reChIP-seq) in order to define the global binding pattern of co-occupied RXRα and RARγ chromatin sites to predict heterodimer binding patterns [46, 60, 61]. Using such strategies, the complexity of RAR/RXR heterodimers can be deconvoluted to reveal the contributions of different receptor pairs. As these studies go forward, it will be important to remember that RXR heterodimers with partners other than RARs may be involved in regulating networks that are initially set up by *at*-RA.

Pioneers, Epigenetic Modifications, and Co-Regulators Establish Regulatory Principles Affecting RA-Regulated Gene Programs Upstream and Downstream of RAR/RXR Heterodimer Action

It is well established that at given times and in particular cells, TFs bind to only a small fraction of their possible target sites in the genome. Pioneer TF remodelling and epigenetic chromatin modification can regulate TF access to certain chromatin sites (for a recent review see [73]). RAR/RXRs themselves may pioneer, for example in the context of RA-induced differentiation, the sub-programs regulated by other TFs. The interplay between the epigenetic status of target gene chromatin and RA regulation has been demonstrated in gene-centric studies with Polycomb proteins and the H3K27me3 mark [1, 25, 34]. Other epigenetic modifications may also regulate receptor recruitment and/or access, and the epigenetic action of co-activator/co-integrators recruited by liganded RAR/RXR heterodimers may exert pioneering activities for downstream programs. Comprehensive analysis of multi-dimensional omics-derived information together with bioinformatics tools that retrieve and integrate data describing RAR/RXR chromatin binding patterns, epigenomes, and transcriptomes will elucidate dynamic gene regulatory networks and provide a framework for experimental confirmation of the molecular mechanisms, key factors, and decision points that define cell fate decisions brought about by RA signaling.

The Dynamic Role of Three-Dimensional Chromatin Organization

The designation of RA target genes from ChIP-seq studies is generally based on proximity criteria which define genes 5 kb or 10 kb away from receptor-binding sites as candidate target genes. Using this definition, a large majority of binding sites are located in intergenic regions, and thus, only a small fraction of all identified binding events are considered in subsequent analyses. The function of these intergenic binding sites has become much clearer from recent studies interrogating the 3-dimensional organization of chromatin in the nucleus. It is now well accepted that the chromatin architecture, i.e. the organization of chromatin in *loops*, *domains* and possibly, *factories* with dedicated functionalities [64], corresponds to a structural organization that regulates the physical interaction between promoters and distant regulatory elements, sometimes with the involvement of non-coding RNAs. This view suggests the entire nucleus to be considered as a regulatory network of its own [20]. Technologies have been developed to analyze this architecture globally [Circular chromosome conformation capture [15, 62, 77]; Hi-C [31, 51, 68]; TCC [32], or with emphasis on a particular signaling or regulatory/processing component [ERα [21]; CTCF [28]; RNA polymerase II [39]. Yet, the dynamic aspect of nuclear architecture in processes like RA-induced differentiation or the changes in nuclear architecture associated with pathologic effects on signaling in diseased cells or organs has not been addressed. It is interesting to note that links between chromatin architecture and features of cancer cells are emerging [55, 58].

Computational Challenges for Omics Data Processing and Integration

The rapid development of next-generation sequencing (NGS) technologies poses multiple challenges for the bioinformatics analyses of enormous amounts of data that are being gathered in massive parallel sequencing projects. At the level of data gathering one of the critical issues, which is still largely ignored in the field, is the need for a generally applicable numerical quality control system; such a system is prerequisite for multi-dimensional data analyses (for a discussion and a recently developed quality control system see [47]. While several computational efforts have aimed to assess the local enrichment confidence in the single NGS-generated profiles that have been reported (for a recent comparison of peak finding algorithms see [45], methodologies for multi-profile comparisons are still in their infancy [17, 27]. The use of integrative genomics approaches may become the methodology of choice for decorticating RA-driven signal transduction events and thus, there is a real need to develop and standardize computational methods with a focus on enhancing the confidence factor in omics datasets. Importantly,

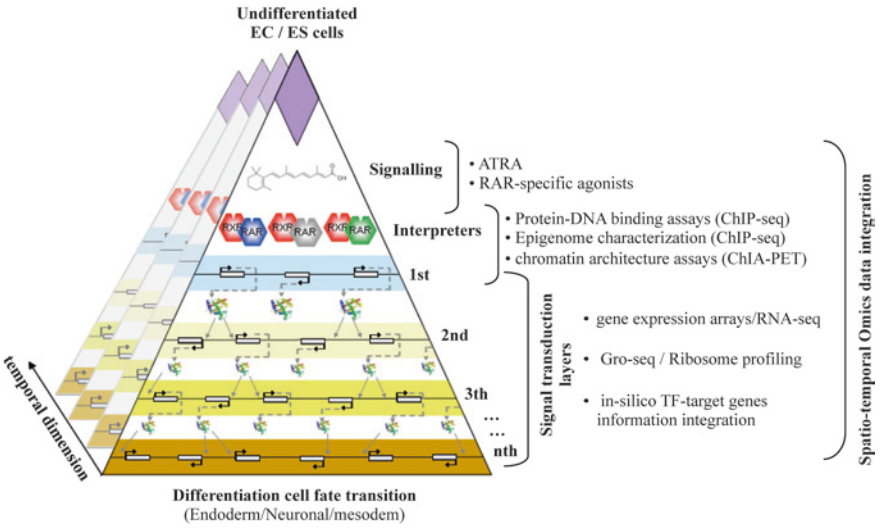

Fig. 9.6 Schematic overview of the spatio-temporal omics data integration designed to study the RA-induced signaling pathway diversification. From *top* to *bottom* The signal induction applied to undifferentiated Embryonic carcinoma (EC)/embryonic stem (ES) cells (ATRA or RAR-specific agonists) is diversified through the interpreters (RXR/RAR nuclear receptors) which may activate several signal transduction layers giving rise to the corresponding differentiation stages. The methodology in use for assessing the presence of the different components involved in this process is displayed in a side. Importantly, two major axes are taken for this analysis: (i) the three-dimensional chromatin structure assessed by proximity-ligation based methodologies (like ChIA-PET) and (ii) the assessment of these events at different time-points during differentiation

future dataset integration in RA-driven differentiation studies will be performed by integrating two major additional elements: (i) the three-dimensional chromatin structure revealed by methodologies like Hi-C or ChIA-PET (see above) and (ii) the temporal nature of the evaluated events throughout the induction process. Importantly, such spatio-temporal analyses will integrate information coming from RAR/RXR binding to chromatin, the chromatin modification status and nucleosome occupancy, and the observed differential transcriptional/translational activity. In addition, computational methods for reconstructing the dynamic regulatory gene networks will be applied with the hope of inferring the temporally defined regulatory decisions that underlie diversification of *at*-RA-induced signaling during developmental processes [14, 18, 48]. Studies done to date have provided initial insight into the enormous complexity that we are facing in stem cell model systems. These data are summarized in a schematic illustration depicting a current view of the molecular and mechanistic 4-dimensional hierarchies (Fig. 9.6) that govern cell fate transitions initiated by a single inducer.

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