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Review

Genome-wide studies of nuclear receptors in cell fate decisions☆

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ABSTRACT

Nuclear receptors (NRs) are important mediators of the information encoded in the chemical structure of its corresponding ligand, as they interpret such information in the context of the cell identity and physiological status and convert it into sequential transcription regulatory events. At the cell level this can result in temporally coordinated processes such as cell fate transitions, which comprise the regulation of a plethora of gene programs including among others regulation of cell proliferation, metabolism and specific functionalities that are acquired by the differentiated cell. While both the early steps of nuclear receptor function and their impact on animal/organ physiology is rather well understood, little is known about the dynamic gene networks that ultimately cause a particular (cell) physiological phenomenon induced by the cognate NR ligand/hormone.

Thanks to advances in massive parallel sequencing and bioinformatics analyses of genome-wide data sets, time has come for the development of NR systems biology. Indeed it is now possible to integrate global transcription factor binding, epigenetic chromatin histone and DNA modification patterns with transcriptomes and 3-dimensional chromatin structures, extract decision points in temporal studies and decipher the temporal control of gene networks that are the ultimate genetic readouts of NR ligand-induced physiological phenomena. In this review we will summarize the chronology of the development of increasingly larger data sets for NR action, with a particular focus on studies performed with the RAR/RXR nuclear receptor family, and discuss the present attempts to integrate a multitude of genome-wide data sets in the ultimate context of the temporal 3-dimensional chromatin structure.

Contents

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1. Introduction

Nuclear receptors (NRs; 48 NRs exist in humans) constitute a major class of transcriptional regulators in metazoans that are believed to have evolved prior to the divergence of vertebrates and invertebrates. The first receptor (estrogen receptor, ER) was identified 1958 by Elwood Jensen but only after cloning several NRs in the 1980s it became apparent that these receptors for steroids, thyroids, retinoic acids and other small molecule ligands, several of which act in an intracrine fashion, constitute a superfamily of transcription factors (TFs) that includes receptors for which no natural ligand is known or may not exist. NRs bind in a ligand-dependent (e.g., estrogen receptor) or ligand-independent (e.g., retinoic acid receptor) manner to *cis*-acting DNA regulatory elements, which may be positioned proximal to promoter regions of target genes or regulate genes due to structural proximity in the context of chromatin architecture, act as activators and/or repressors of transcription, and may exert non-genomic activities. NRs are of major social (the "pill", NR antagonists for abortion), pharmaceutical and clinical importance (e.g., endocrine-dependent cancers or metabolic diseases), as a large majority of physiological processes and pathologies involve (aberrant) NR action [for reviews see 1,2–13].

Conceptually, ligand binding modulates the communication of the nuclear receptor with the intracellular environment, which entails essentially receptor-protein and receptor-DNA or receptor-chromatin interactions. During this process, receptors are important mediators of the information encoded in the chemical structure of a given ligand, as they interpret this information in the context of cellular identity and cell-physiological status; thus transforming it into a dynamic chain of receptor-protein and receptor-DNA interactions. NRs present a modular structure mainly characterized by a DNA-binding (DBD) and ligand-binding (LBD) domains, whose 3D structures in presence and absence of cognate DNA response elements and various agonists or antagonists, respectively, have been determined [14–19]. The LBD serves as dual input–output information processor, as ligand binding (other inputs are, for example, receptor phosphorylations) induces allosteric changes of receptor surfaces that represent docking sites for subunits of transcription and/or epigenetic machineries, or enzyme complexes (output). Furthermore NRs are actively regulated by post-translational modifications (e.g., phosphorylations; ubiquitinylations) which may have a direct or indirect role in their transcriptional regulation function (reviewed in [20]).

Both the early steps of nuclear receptor function as well as their physiological impact are in general rather well understood. In fact, due to a plethora of molecular and structural biology studies, the sequence of events that follows the binding of a ligand is largely known, and we understand how these events can be modulated by ligand design [3,21,22]. Briefly, binding of an agonist to the NR ligand binding domain (LBD) induces an allosteric conformational reorganization which alter surfaces in the ligand-free receptor (apoNR) to which co-repressors (CoRs) bind resulting in dissociation of CoR complexes, which contain epigenetic enzymes (HDACs, histone deacetylases). Importantly, only some apoNRs, such as RARs and TRs, recruit CoR complexes and thus, can act as transcriptional repressors when binding to chromatin in absence of ligand. Other NRs, like ER and GR are believed to bind to chromatin only after interaction with their corresponding ligands.

In addition to the molecular/structural insights, extensive mouse genetics provided important information concerning the physiological roles of several nuclear receptors [23,24], and of some of their co-regulators [25,26]. However, how a single molecule that binds to its corresponding receptor 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) physiological phenomenon, is still a unknown (Fig. 1). In this review, we summarize the efforts performed to pave the way into the development of systems biology of nuclear receptor action. We will address the chronology of the development of increasingly larger omics data sets for NRs action, with a particular focus on the RAR/RXR nuclear receptors, and discuss the present attempts to integrate a multitude of genome wide data sets in the ultimate context of the 4-dimensional structure of chromatin.

2. Nuclear receptors in a post-genomic era

The publication of the first draft of the human genome sequence in 2001, followed by those of various other model organisms, gave rise to a new way to address the molecular genetics of the homeostasis of living organisms. Since then, any biological phenomena and its (de)regulation can be explored, in principle, in a "genome-wide" context. Indeed, thanks to the advances in genome-wide or "omics" approaches, it is now possible to assess the global transcriptional activity by a variety of approaches (i.e., by microarrays; RNA sequencing, etc.), characterize the genomic localization of transcription factors or evaluate epigenetic chromatin modification in a high resolution manner (ChIP-chip; ChIP-seq assays). Furthermore, new methodologies using a proximity-based ligation approach are starting to give insights into the 3-dimensional chromatin structures providing a new way to interrogate the molecular principles regulating living systems, such as the gene networks involved in cell fate decisions that are triggered by internal or external factors (Fig. 1).

2.1. Assessing the global gene expression signatures driven by NRs

The dissection of NR ligand-induced signaling involved in various physiological processes has been early on evaluated by high throughput genomic methods. Importantly, this new way to interrogate the molecular homeostasis of biological systems generates higher number of significant targets than those identified in previous years by standard genetics/molecular biology approaches, thus providing a more comprehensive view of the regulatory events during NR-signaling. Indeed, in 2002 Balmer and Blomhoff summarized more than 1191 published articles on retinoic acid receptors and classified 532 genes as RA regulated targets [27]. In the same year, two other studies focused on RA-induced cell differentiation in two well known embryo carcinoma cell (ECC) models (F9 differentiates into parietal endoderm [28], while P19 differentiates into neuronal cells [29]) identified a similar number of differentially regulated genes by incorporating in their assays one of the early versions of the microarray technology (cDNA PCR-spotted microarrays; reviewed in [30]). Furthermore, these two studies as well as others focused in the global transcriptional regulation response driven by various other NRs (an extensive review concerning the use of microarrays for the genomic profiling in a NR-mediated context has been presented in [31]), assessed the changes in transcriptional activity over different time-points as a way to identify gene-specific signatures as well as temporal associations, paving the way towards a spatio-temporal view of cell-fate transitions (further discussed in Section 3).

Despite the important number of NR-regulated genes identified in these studies, the major limitation of this approach was that direct NR targets could not be distinguished from genes that were indirectly regulated. To partially circumvent this problem, the use of protein synthesis inhibitors, like cycloheximide, was introduced during the assays to avoid transcriptional regulation cascades progression. In this context, Harris et al. induced F9 differentiation during 6 h in presence of ATRA and the protein inhibitor cycloheximide which gave rise to the identification of 109 significantly

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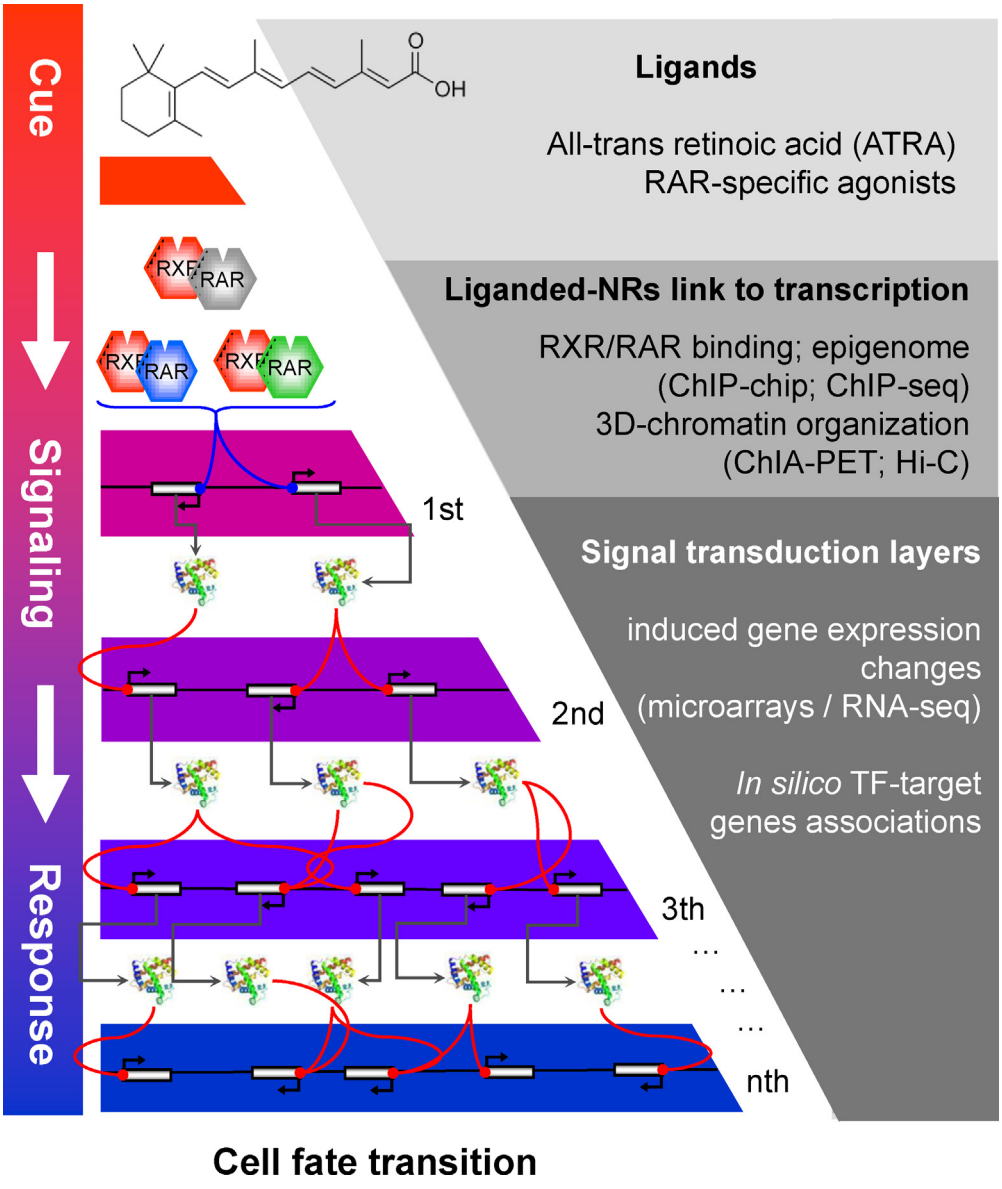

Fig. 1. Schematic representation of the retinoic acid signaling transduction process assessed by global approaches. From top to bottom: cell fate transition is a consequence of an initial cue [all-trans retinoic acid (ATRA) and related retinoid ligands] that initiates signal transduction through transcription regulation primarily mediated through the corresponding RAR/RXR receptors. The initial signal transduction cascade is diversified over several signal transduction layers, which together specify the associated cell fate transition. Both the primary signaling response, as well as the further signaling transduction layers can be evaluated through global approaches (right side of the panel). Methods listed for assessing the 3D-chromatin organization corresponds to Hi-C (High resolution chromatin conformation capture [74]) and ChIA-PET (Chromatin Interaction Analysis by Paired-end tags sequencing [72]).

differentially regulated genes [28]. While the use of such inhibitor appears as an elegant way to identify in a selective manner primary/direct targets, the study performed in the RA-induced F9 differentiation system demonstrated that only 22 of them were present in the ATRA control assay, suggesting that the other genes are artifactually induced by cycloheximide treatment.

Further global transcriptome studies performed in different model systems incorporated systematically early and late treatment time points, under the assumption of a direct correlation with putative primary/direct and secondary/indirect NR-responsive genes [32]. Others took advantage of the availability of specific ligands as a way to restrict the analysis to a given set of differentially regulated genes. For instance, the synthetic pan-RAR agonist TTNPB has been used to decrease the 'contamination' with genes responding to permissive RXR heterodimers, as all-trans RA isomerizes to the RXR ligand 9-cis RA [33,34].

Considering that NRs can be expressed as different isotypes/isoforms, evaluating their specific transcriptional regulation cascades through global approaches became a crucial task for understanding the biological role of NR diversity. In the particular case of the RA nuclear 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 [1]. While all three RAR isotypes were shown to be present in model systems like the F9 ECCs, previous studies had already provided evidence for specific roles of such isotypes; for instance RXRα/RARγ heterodimer isotype is essential for RA-induced F9 differentiation [35–37].

In this context, Su and Gudas aimed at identifying the specific role of RARγ by performing global gene expression profiling with wild-type and RARγ−/− F9 cells in presence or absence of ATRA [38]. While they demonstrated that wild type and the RARγ−/− cells presented similar morphological and proliferation

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characteristics in the absence of RA treatment, important gene expression differences were observed; this reveals limitations for the use of RAR knockout cells to decipher specific roles of RAR isotypes. Similar observations made for global transcription regulation studies performed in RARα−/− F9 cells [39] and in a gene-centric manner for several RXR and RAR isotype knockouts [35,36] revealed "artifactual" ligand responses of specific RXR–RAR heterodimers thus suggesting that global studies with RXR–RAR knockout cells need to be interpreted carefully.

In summary, global approaches for assessing the changes in gene expression regulation in the context of a liganded-NR function were widely used in the past providing a rather fast and accurate way to identify gene expression signatures in the studied systems. Nevertheless, while these studies identified differentially regulated genes, they did not provide insights into the gene programming functions of liganded NR(isotype)s.

2.2. Mapping the chromatin binding sites of nuclear receptors

NR signaling is based on their capacity of regulating transcription; thus the dissection of the effects of NRs on physiological processes requires the comprehensive mapping of their dynamic interactions with the chromatin to identify regulated target genes. As the DBD is the primary determinant of DNA interaction specificity a large number of studies has been devoted to the understanding of the sequence-specific and structure determinants of NR DBD–DNA interaction ([40–44]; for a list of NR binding sites see [45]). In the case of RA receptors, in vitro binding and transactivation studies demonstrated that RXR/RAR heterodimers bind preferentially 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 nucleotide (DR5, DR2, DR1) due to the dimerization characteristics of the DNA binding domain [41–43,46]. This characteristic RA-Responsive Element (RARE), has been shown to present major divergence when compared with the RXR/RAR binding sites associated to well-known RA-induced genes [47], indicating that the consensus RAREs may correspond to high affinity binding sites but occur rarely in natural RA target genes. Indeed, high affinity binding sites can be isolated by co-immunoprecipitation with DNA but those sites are not used for gene regulation, most likely because they are not accessible in the corresponding chromatin [48]. Furthermore, mapping the potential RXR/RAR binding sites by comparing the consensus RAREs does not take in consideration additional mechanisms, like the epigenetic mechanisms that regulate access of RXR/RAR heterodimers [49,50] and steroid receptor homodimers [51], and/or the synergistic interaction with other NR/TFs [52].

For these reasons, the current method of choice for comprehensive and unbiased mapping of the protein-chromatin interactions is the use of chromatin immunoprecipitation combined with high throughput sequencing. Note that the hybridization of immunoprecipitated chromatin to microarray chips (known also as ChIP-chip) represented the first approaches for global mapping of NR-chromatin interaction. In fact, Delacroix et al. aimed at identifying the RAR binding sites to discriminate between direct and indirect RA-regulated targets using Taf4$^{lox/-}$ MEFs, which undergo morphological changes upon RA treatment accompanied by regulation of >1000 genes [53]. After integration of 3xFlag-HA tagged RARα or RARγ isotypes they performed ChIP assays and hybridized the IPed DNA to Agilent promoter microarrays (ChIP-chip) [54]. Surprisingly, they identified ~300 RAR-occupied sites of which <25% corresponded to differentially RA-regulated genes. Later studies performed in various other systems demonstrated that only an small fraction of the RXR/RAR binding sites are located in promoter regions, thus explaining, at least partially, the low correlation between RAR occupancy and gene expression regulation observed in this study.

In fact, using a conceptually similar approach, Hua et al. have integrated eGFP-tagged RARα or RARγ into human MCF-7 breast cancer cells to characterize the RAR isotype-selectively regulated pathways implicated in the anti-proliferative and apoptotic effects of RA [55]. Importantly, the RAR-specific IPed chromatin was hybridized to tiling arrays containing more than 40 million oligonucleotide probes virtually interrogating the entire human genome. Under these conditions, they found >3000 RARγ and >7000 RARα binding sites respectively, from which more than 85% of the identified sites were located in intronic or promoter-distal intergenic regions.

In a similar manner a recent study with mouse embryonic stem cells aimed at identifying the RA-dependent gene programs involved in neuronal differentiation [56], but in contrast to the previously mentioned studies, the binding of endogenous RAR was mapped by using a pan-RAR antibody and combining ChIP assays with massive parallel sequencing (ChIP-seq); thus they avoided over-expression and/or "artefactual" binding of tagged constructs. This assay, performed before and after 8 h of ATRA treatment, revealed both constitutive and de novo binding sites upon RA exposure, which were then correlated with global microarray-based gene expression and RNA polymerase II initiation and elongation (assessed by ChIP-seq). The fraction of differentially regulated genes associated with RAR binding was estimated by using a 5 kb proximity criterion; this way only 15% of the identified binding sites could be linked to a (transcriptionally active) coding region. Indeed, it is now generally accepted that distal enhancers, which cannot be identified by simple binding site proximity, can regulate NR-responsive genes (further discussed in Section 3.2.3).

Our own recent study used the well-established F9 model to dissect the gene regulatory pathways that are responsible for the RA-induced endodermal differentiation by integrating the global RAR binding and gene regulation information from five different time-points during the first 48 h after RA exposure [57]. For each time-point the differential transcriptional regulation has been assessed after treatment with ATRA or RARα, β, γ-specific agonists.

Given the essential role of RARγ in F9 cell differentiation [37], we inferred the RXRα–RARγ heterodimer-genomic location by mapping both heterodimer components separately at all 5 time-points (Fig. 2A). Overall, RXRα displayed more binding sites than RARγ, as was expected from the promiscuous heterodimerization of RXRα with multiple partners. When evaluating RXRα and RARγ binding sites as heterodimer components, we identified a constitutive RXRα–RARγ binding population plus another presenting a highly dynamic behavior during ATRA treatment (Fig. 2B). In fact, while the overall numbers of RXRα–RARγ binding sites decreased during F9 differentiation (~2000 sites in the absence of treatment and less than 1000 sites after 48 h in presence of ATRA) (Fig. 2B and C), we detected significant amounts of de novo recruited heterodimers even after 24 or 48 h of treatment, indicating sustained and highly dynamic interaction of the RXRα–RARγ heterodimer with chromatin targets during this cell physiological process. Unexpectedly, the overall decrease of RXRα–RARγ heterodimers binding sites did not correlate with the observed amounts of RXRα binding sites, thus suggesting that the observed decrease of the binding sites of RXRα–RARγ heterodimers may result from an exchange with other RXRα heterodimers during the process of differentiation (Fig. 2C).

Comparing RA-induced gene expression with the receptor binding in a 10 kb distance interval (Fig. 3A) we found that more than 50% of genes induced during the first 24 h of ATRA treatment showed a RXRα or an RXRα–RARγ binding site within 10 kb

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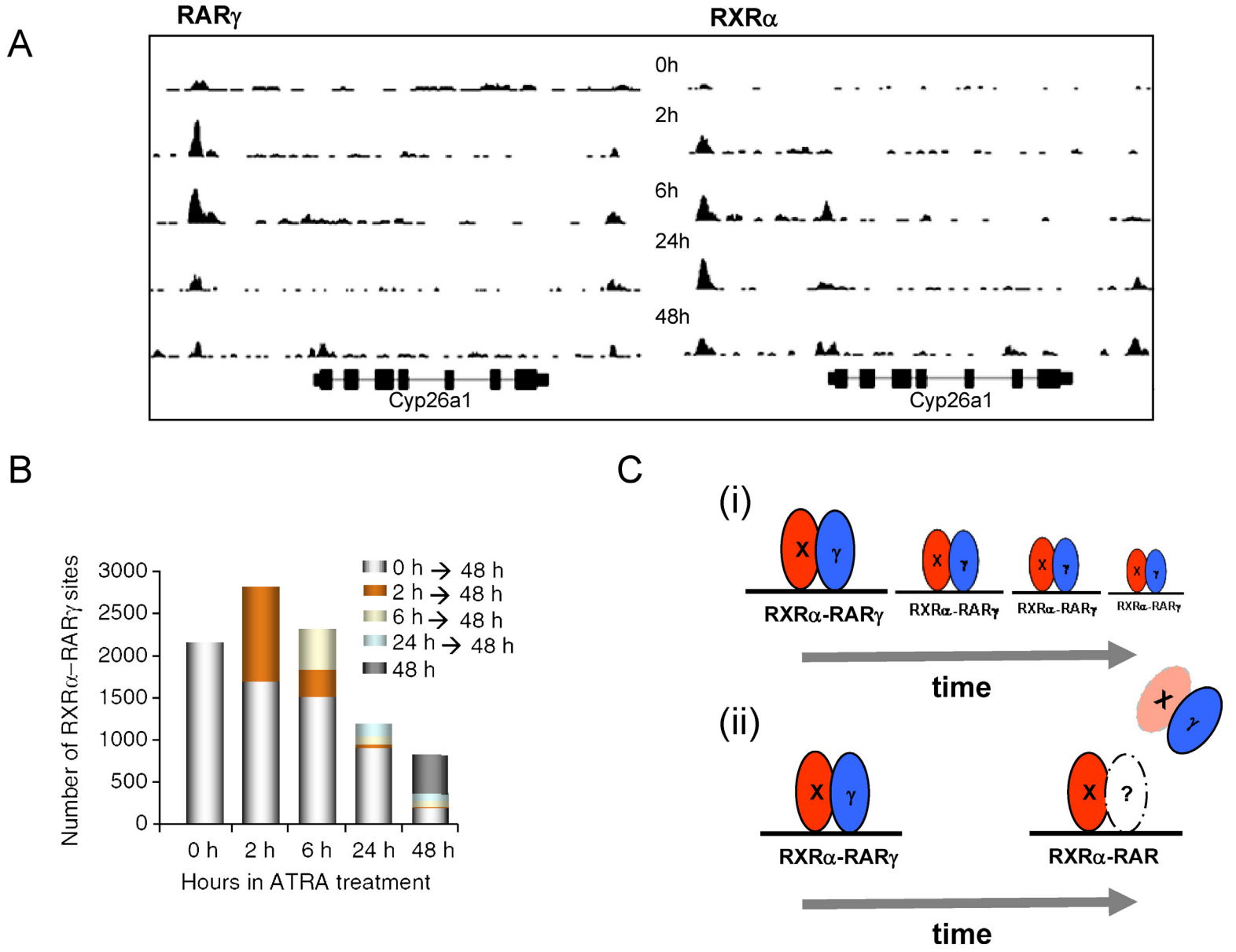

Fig. 2. RXRα and RARγ nuclear receptors present a highly dynamic binding to chromatin during ATRA-induced F9 differentiation. (A) Temporal recruitment of RARγ and RXRα in proximity of the Cyp26a1 locus, as revealed by ChIP-seq. Note that both receptors bind to the identical chromatin locus but with different dynamics. (B) The number global RXRα–RARγ binding sites (defined by the co-occurrence of both receptors) are illustrated in the context of their temporal recruitment, duration of occupancy and dissociation. RXRα–RARγ co-occupied sites per time point are sub-classified based on their recruitment intervals and depicted by color coding. (C) Schematic model illustrating the (i) global progressive loss of RXRα–RARγ heterodimer as observed in figure (B); as well as (ii) that of RARγ but not of RXRα from chromatin binding sites observed during ATRA-induced F9 differentiation; accounting for an unexpected dynamic pattern associated to the heterodimer composition.

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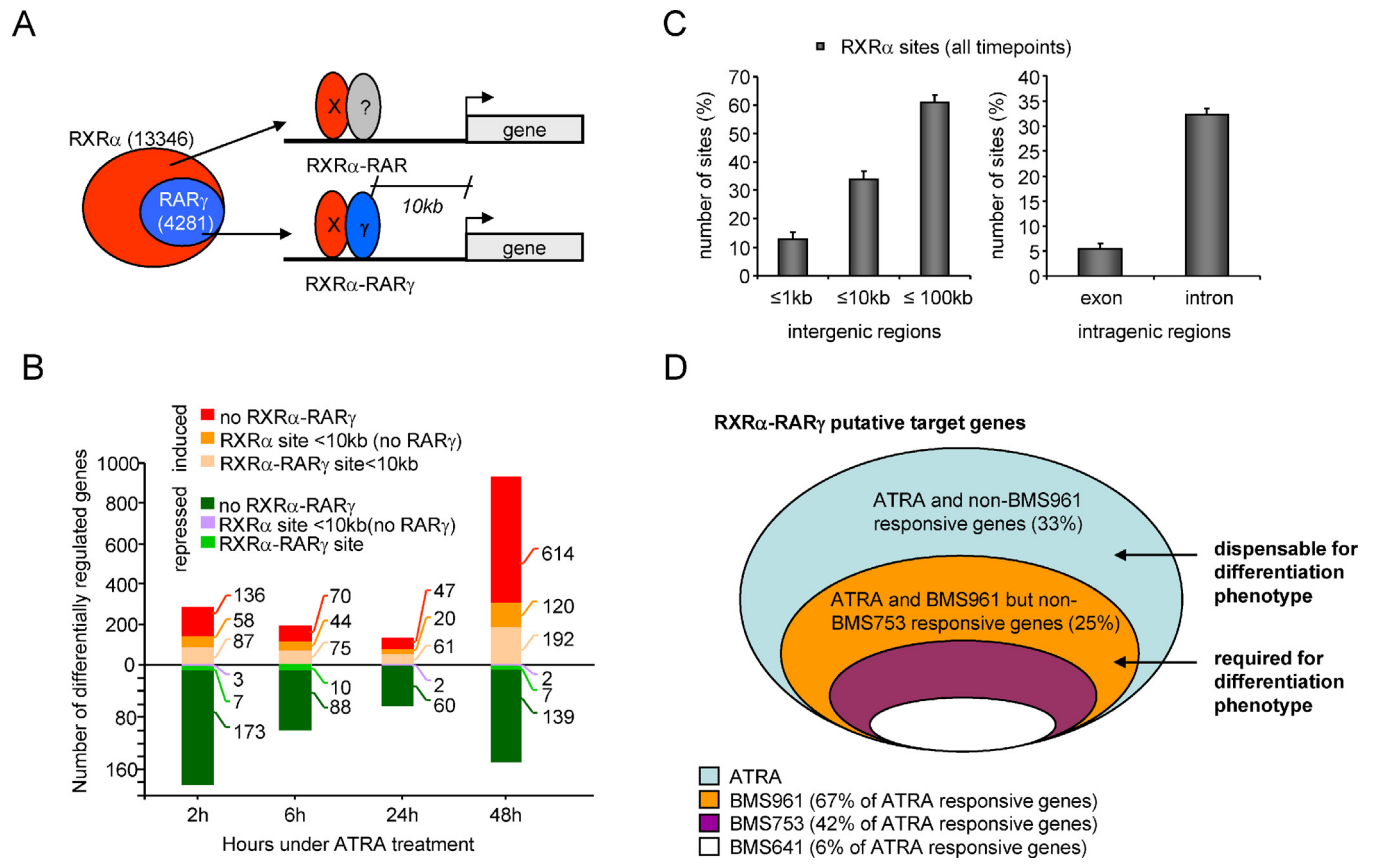

Fig. 3. Differential gene expression induced by retinoids in F9 embryonal carcinoma cells. (A) Schematic representation illustrating the transcription regulation activity associated to the proximal localization of RXRα–RAR nuclear receptors. Note that genes were classified as putative target genes if at least one RXRα or RXRα–RARγ binding site was located in up to 10 kb distance. (B) 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 following the criterion illustrated in (A). (C) RXRα binding sites classified in their genomic context demonstrates that more than 70% of them are located far away from coding regions (>10 kb distance; left panel), in addition to their strong preference for intergenic regions (right panel). (D) RXRα–RARγ ATRA-putative target genes described in (B) were further classified based on their response to RAR-specific agonists. Taken in consideration that only the RARγ agonist (BMS961) can reproduce the F9 differentiation phenotype observed during ATRA treatment, the characterized RXRα–RARγ ATRA-putative target genes were further classified as "dispensable" and "required" for inducing the differentiation phenotype.

proximity (Fig. 3B). 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 (Fig. 3C), suggesting that they might regulate transcription through 3-dimensional chromatin structures or may regulate as yet non-annotated transcripts. To further confirm the direct transcriptional regulation by the characterized RXRα–RARγ binding sites, we compared the transcriptional responses in presence of ATRA or RAR-specific agonists [57]. Importantly, 67% of the ATRA-induced putative RXRα–RARγ targets did respond similarly to the differentiation competent RARγ agonist BMS961. Surprisingly, also the treatment with BMS753 (RARα-specific agonist) or BMS641 (RARβ-specific agonist) induced a response of some ATRA-RXRα–RARγ targets, albeit only in a minor fraction (42% and 6% of the ATRA-induced genes, respectively). This suggests that among the characterized ATRA-dependent RXRα–RARγ targets (i) ~30% of them are dispensable for inducing the observed cell differentiation phenotype; and (ii) from the remaining 70% only a third of them are indeed essential for driving the differentiation process (Fig. 3D). Importantly, inside this last population comprises several TFs, like Foxa1, Foxp1, Hoxa5, Hoxb5, Rarb or RXRγ, indicating that RA-signal transduction invokes the induction of "downstream" transcription factors, which in turn regulate signaling bifurcation events to yield the final differentiated phenotype.

3. NR gene expression programs and their associated key factors involved in signaling diversification

3.1. Studying NR-driven cell fate transitions as dynamic gene expression programs

As mentioned above, the integrative analysis of global gene expression response to a given ligand and the corresponding NR-chromatin association can, in principle, identify an important proportion of the NR-mediated gene-regulatory events. Furthermore, the use of dynamic binding and transcription information provided additional insight in molecular mechanisms occurring during cell-fate transition. In fact, these studies revealed (1) a highly dynamic target gene expression [28,29,57] and (2) similarly dynamic chromatin occupancy of pre-existing and de novo recruited RXR–RAR heterodimers, including heterodimer replacement or even heterodimer partner swaps [57]. Similar studies performed in other model systems like 3T3-L1 cells integrated the global chromatin localization of RXR and PPARγ NRs with that assessed for RNA polymerase II during the induction of adipocyte differentiation [58]. Like in the previously discussed RA-induced F9 differentiation study [57], this integrative analysis performed in a temporal manner revealed a differential recruitment of PPARs and RXR during adipogenesis and allowed the classification genes by their relative transcriptional activity assessed from

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RNA polymerase II binding and the presence of RXR/PPARγ in proximity.

Despite these findings, the gain of information by this type of integrative omics analyses remains restricted to directly characterize the corresponding NR-regulated targets and their potential temporal changes, which represents only a small fraction of all differentially regulated genes. NR-driven cell-fate transitions are expected to take place through a signal transduction mechanism, 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 the signaling at different levels [57,59], which ultimately leads to the emergence of a specific cell phenotype/functionality.

The firsts signal transduction layers are mediated mainly, albeit not exclusively by transcription factors (TFs). While the reconstruction of the executor programs may profit from the characterization of the cascade of TFs that propagate the signal transduction and diversification process, it is virtually impossible to directly characterize these events in a given system by applying series of ChIP-seq assays targeting all potentially related TFs. Nevertheless, the availability in public repositories of TFs interactomes for multiple cell models represents an important resource for in silico dataset integration; note in this respect the important contribution by the ENCODE consortium [60].

In fact, the deconvolution of the RA signaling pathways during the F9 induced differentiation has been performed by integrating TF target gene annotations, including the identified direct putative RXRα–RARγ targets, with the ATRA-induced gene programming [57]. This analysis, performed with the Dynamic Regulatory Events Miner (DREM; [61]), predicted six distinct gene co-expression paths, which recapitulate the different subprograms generated during the RA-induced signal transduction. In addition to classify the temporal gene expression information in co-expression paths, DREM evaluates whether a given co-expression path is enriched for genes that are annotated as targets of a specific TF, whose action contributes to the predicted bifurcation. In this manner, DREM predicted 3 bifurcation points leading to signal diversification and associated to candidate TFs. As proof-of-principle, DREM associated RXRα–RARγ with upregulated subprograms validated by differential gene expression and the chromatin-binding pattern of RXRα–RARγ (Fig. 4). Notably, DREM predicted transcription factors of the Homeobox family (e.g., Hoxa1, Hoxb2, Hoxb4, Hoxb5) and others like RARα or Foxa2, to be enriched in the upregulated subprograms, while the repressed path was associate with TFs like Egr1 [62] and Sox2 [63], previously described as positively regulating cell proliferation and stem cell pluripotency.

The predicted RA-induced co-expression paths were further evaluated in the context of gene co-citation interactions to construct the RA-driven RXRα–RARγ-mediated signaling network. This type of analysis provides a global view of the relevant genes involved in signal transduction by integrating information extracted from the existing literature on previously reported interactions and provides a comprehensive way to associate functional features to the predicted subprograms. Importantly, this analysis illustrates the complex temporal coordination of the variety of molecular processes involved in RA-induced differentiation and predicts critical nodes associated with the cell fate transition initiated by RA [57].

3.2. Additional mechanisms involved in controlling the retinoids-driven dynamic diversified gene programs

3.2.1. Multiple RXR–RAR heterodimers mediate RA-signaling

As mentioned above, the first level of signal diversification results from the multiplicity of RXR–RAR complexes that can be formed depending on the actual expression levels of 6 receptor isotypes (RXRα; RXRβ; RXRγ RARα; RARβ; RARγ). An enigmatic aspect characterized in our study concerns the highly dynamic binding and the potential "heterodimer components swapping" of RXRα–RARγ heterodimers during the RA-induced differentiation process. While the methodologies allowed highlighting such phenomenon, its biological significance remains elusive. Clearly, exploring the role of other RXR/RAR heterodimers during this process will provide insights into the heterodimer cross-functionalities as basis for the observed dynamics.

Such studies necessitate reChIP assays to provide reliable information about co-occupancy of the evaluated heterodimer partners at a given chromatin site. While reChIP assays were previously shown as a powerful method for evaluating simultaneous co-occupancy events in a locus-centric manner [64,65], their low yields are not compatible with the requirements for global ChIP-seq assays. To overcome this problem we have recently combined reChIPs with linear DNA amplification (LinDA-reChIP-seq) in order to define the global binding pattern of co-occupied RXRα and RARγ chromatin sites to predict heterodimer binding patterns [66–68]. Using such strategies, the complexity of RXR–RAR heterodimers can be decorticated toward the contributions of the different combinations of receptors.

3.2.2. Epigenetic modifications and co-regulators establish regulatory principles affecting RA-regulated gene programs upstream and downstream of RXR–RAR heterodimer action

In addition to the TF-driven decisions for signal diversification process during RA-induced differentiation, several additional factors and regulatory paradigms may impact on program execution. In fact, epigenetic modification of chromatin and its interplay with RA regulation has already been demonstrated in gene-centric studies with Polycomb proteins and H3K27me3 [49,69,70]. Other epigenetic modifications may also regulate NR's recruitment and the epigenetic action of co-activator/co-integrators recruited by liganded RXR/RAR heterodimers may exert pioneering activities specifying downstream programs.

A novel mechanism of signaling pathway diversification has been reported recently by Ceschin et al. [59]. Studying estrogen receptor (ERα) signaling in breast cancer cells they analyzed the role of two epigenetic factors, the histone acetyltransferase (HAT) CBP and the methyltransferase CARM1/PRMT4. Both CBP and CARM1 bind to the SRC/p160 co-activators and are co-recruited by agonist-bound ERα to chromatin targets. Based on previous knowledge that CARM1 methylates CBP at specific arginine residues [71] and the observation that CBP methylation was exclusively CARM1-dependent, they mapped not only the binding site repertoires of ERα, SRC3, CBP, CARM1 and acetylated histones (H3K18ac) but also the methylated CBP species using antibodies that recognize selectively the methylated CBP residues [59]. Interestingly, the first observation was that methylation at R2151 was required for estrogen-dependent recruitment of CBP to chromatin. The molecular basis for this requirement is not known and it is unclear if this is a general phenomenon or restricted to certain cell (types). Moreover, the subsequent multi-dimensional analysis, which included a time-series of transcriptomics, identified distinct "hubs" of ERα target genes. These hubs differed by the recruitment of the particular methyl-CBP species (which vary in HAT activities), thus indicating that the crosstalk between co-recruited epigenetic factors can lead to pathway diversification. It will be interesting to assess whether these different hubs, as well as those formed by the other HAT p300 correspond to functionally related target genes and whether a "methyl-HAT code" may indeed exist.

The above studies show that comprehensive multi-dimensional omics-derived information together with the bioinformatics tools to define dynamic gene regulatory networks by integrating NR

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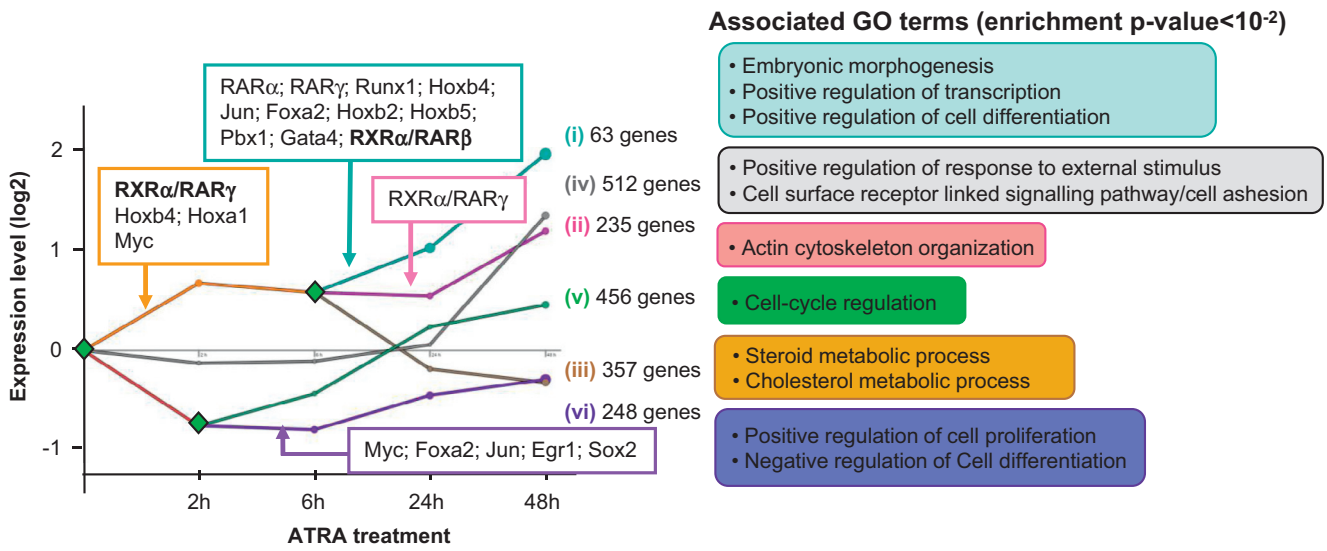

Fig. 4. Dynamic regulatory map of ATRA-induced transcriptome. DREM co-expression analysis; color-coded paths summarize common characteristics. Diamonds indicate predicted bifurcation points which give rise to the different co-expression paths; transcription factors whose target genes are over-enriched in a given path are illustrated. The number of genes per co-expression path, as well as their relevant Gene Ontology terms is displayed on the right.

chromatin binding patterns, epigenomes and transcriptomes will shed significant light on the molecular mechanisms, key factors and decision points that define decisions specifying cell fate and cell function.

3.2.3. The three-dimensional chromatin organization and its dynamic changes driven by NR-signaling

The designation of NR target genes from ChIP-seq studies is generally based on linear proximity criteria. However the large majority of binding sites are located in intergenic regions and thus, only a small fraction of all identified binding events are generally considered in such 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; for instance in the particular case of ERα [72]. It is now generally accepted that the chromatin architecture, i.e., the organization of chromatin in "loops', 'domains' and possibly 'factories' with dedicated functionalities, corresponds to a structural organization that specifies the physical interaction between promoters and distant regulatory elements, sometimes with the involvement of non-coding RNAs. Indeed, the entire nucleus has to be considered as a regulatory network of its own [73]. Importantly, the combination of proximity ligation-mediated assays with massive parallel sequencing provided the technology to analyze this architecture globally (Hi-C [74]; TCC [75]). Furthermore, the incorporation of a prior immunoprecipitation step during these assays allows to study such 3D-chromatin organization in association with a given signaling or regulatory/processing component (ERα [72]; CTCF [76]; RNA polymerase II [77]). Yet, the dynamic aspect of nuclear architecture in processes like RA-induced differentiation or the changes of nuclear architecture in related pathologies, with its subsequent consequences on signaling of the diseased cell/organ, has not yet been addressed. It is interesting to note in this respect that links between chromatin architecture and features of cancer cells are emerging [78,79].

4. Future directions in the study of NR-driven cell fate transition

How can the structural information that is present in a simple chemical molecule, like all-trans retinoic acid (ATRA), be 'read' to set-up the sequence of temporally controlled events, which finally lead to the cell-physiological changes that characterize a differentiated cell? Our previous study performed with the embryo carcinoma F9 cell model system provided for the first time a systems biology view of the ATRA-induced signaling pathway diversification through different regulatory decisions characterized at different time-points during differentiation [57]. Yet the view of the retinoic acid (RA)-induced signal transduction events inferred from this study is far from being comprehensive. This is in part due to the reduced number of molecular events that could be imported in the spatio-temporal omics data analyses (discussed above), but also a consequence of technical constraints related to the complexity of the system which operates with up to six receptors and multiple heterodimers.

The rapid development of next-generation sequencing (NGS) technologies poses multiple challenges for the bioinformatics analyses of the enormous amounts of data that are gathered by massive parallel sequencing. While in the past years several computational efforts aiming to assess the local enrichment confidence in single NGS-generated profiles have been reported, a number of key issues concerning methodologies for multi-profile comparisons are lacking or are only incompletely addressed. As discussed above, the use of integrative genomics approaches may become the methodology of choice for decorticating the NR-driven signal transduction events; thus the implementation of suited computational methods focused on enhancing the confidence in omics data assessment at the time of their integration represents an essential aspect to consider for this kind of studies. Importantly, future dataset analyses of NR-driven differentiation studies will need to integrate two major additional elements: (i) the three-dimensional chromatin structure revealed by methodologies like Hi-C (High resolution chromatin conformation capture [74]) or ChIA-PET (Chromatin Interaction Analysis by Paired-end tags sequencing [72]) and (ii) the temporal nature of the evaluated events throughout the induced (cell physiological) process. Importantly, such spatio-temporal analysis will provide information about NR binding within the chromatin architecture, the chromatin modification status and nucleosome occupancy, and the observed differential transcriptional/translational activity in a given physiological context. In addition, computational methods for reconstructing the dynamic regulatory gene networks may be applied with the aim of inferring the ATRA-induced signaling pathway diversification through temporally defined regulatory decisions as illustrated in previous

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studies [57,61,80]. These studies have provided an initial insight into the enormous complexity that we are facing already in model systems, such as stem cells, when trying to understand at a molecular and mechanistic level the 4-dimensional hierarchies that are initiated by a single inducer and govern cell fate transition.

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