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# A System Biology Approach to Identify Regulatory Pathways Underlying the Neuroendocrine Control of Female Puberty in Rats and Nonhuman Primates

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## Abstract

Puberty is a major developmental milestone controlled by the interaction of genetic factors and environmental cues of mostly metabolic and circadian nature. An increased pulsatile release of the decapeptide gonadotropin releasing hormone (GnRH) from hypothalamic neurosecretory neurons is required for both the initiation and progression of the pubertal process. This increase is brought about by coordinated changes that occur in neuronal and glial networks associated with GnRH neurons. These changes ultimately result in increased neuronal and glial stimulatory inputs to the GnRH neuronal network and a reduction of transsynaptic inhibitory influences. While some of the major players controlling pubertal GnRH secretion have been identified using gene-centric approaches, much less is known about the system-wide control of the overall process. Because the pubertal activation of GnRH release involves a diversity of cellular phenotypes, and a myriad of intracellular and cell-to-cell signaling molecules, it appears that the overall process is controlled by a highly coordinated and interactive regulatory system involving hundreds, if not thousands, of gene products. In this article we will discuss emerging evidence suggesting that these genes are arranged as functionally connected networks organized, both internally and across sub-networks, in a hierarchical fashion. According to this concept, the core of these networks is composed of transcriptional regulators that, by directing expression of downstream subordinate genes, provide both stability and coordination to the cellular networks involved in initiating the pubertal process. The integrative response of these gene networks to external inputs is postulated to be coordinated by epigenetic mechanisms.

### Keywords

Female puberty; hypothalamus; transcriptional regulation; gene networks; systems biology; neuroendocrine control; neurotransmission; glial-neuronal communication

## The biological underpinnings of the cellular systems controlling puberty

The basic neuroendocrine mechanisms controlling the initiation of female reproductive capacity are well characterized. An increase in pulsatile release of gonadotropin-releasing

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hormone (GnRH) is ultimately responsible for setting in motion the endocrine manifestations of puberty. This change is, in turn, determined by modifications in transsynaptic (Kordon and others, 1994; Ojeda and Terasawa, 2002) and glial (Ojeda, Lomniczi, and Sandau, 2010; Ojeda, Lomniczi, and Sandau, 2008b) inputs to the GnRH neuronal network. While the transsynaptic changes involve an increase in excitatory inputs and a reduction in inhibitory influences (Ojeda *et al.*, 2002; Plant and Witchel, 2006; Terasawa and Fernandez, 2001), the glial component is predominantly facilitatory, and exerted by growth factors and small molecules that stimulate GnRH secretion (Lomniczi and Ojeda, 2009; Ojeda and Skinner, 2006).

In the last ten years, significant progress has been made towards identifying neuronal subsets involved in both the excitatory and inhibitory control of GnRH secretion. We now know that the excitatory control of puberty is not only provided by glutamatergic neurons (Ojeda *et al.*, 2006; Plant *et al.*, 2006), but even more conspicuously by neurons that produce kisspeptin [reviewed in (d'Anglemont, X and Colledge, 2010; Oakley, Clifton, and Steiner, 2009)]. Kisspeptins are a family of peptides encoded by the *KISS1/Kiss1* gene and that act as powerful stimulators of GnRH release (Kauffman, Clifton, and Steiner, 2007; Oakley *et al.*, 2009; Shahab and others, 2005); in their absence or in the absence of its receptor (known as GPR54 or Kiss1R), puberty fails to occur (de Roux and others, 2003; Lapatto and others, 2007; Seminara and others, 2003; Topaloglu and others, 2012). In primates, kisspeptin neurons are mostly located in the arcuate nucleus (ARC) of the medial basal hypothalamus (Shahab *et al.*, 2005). In rodents, there is a second population of kisspeptin neurons located in the periventricular region of the anteroventral periventricular nucleus (AVPV) (Clarkson and others, 2009; Gottsch and others, 2004). It appears now clear that kisspeptin neurons of the ARC are required for pulsatile GnRH release (Navarro and others, 2011; Wakabayashi and others, 2010), and that at least in rodents AVPV kisspeptin neurons are needed for the pre-ovulatory surge of gonadotropins (Khan and Kauffman, 2011; Smith and others, 2006).

In all mammalian species so far studied the first neuroendocrine manifestation of puberty is a diurnal increase in pulsatile LH release [reviewed in (Ojeda *et al.*, 2006)], which is in all likelihood driven by kisspeptin neurons of the ARC. These cells have been termed KNDy neurons (Lehman, Coolen, and Goodman, 2010; Navarro *et al.*, 2011), because they produce **K**isspeptin, **N**eurokinin B (NKB) and **D**ynorphin (Navarro *et al.*, 2011; Wakabayashi *et al.*, 2010). It is currently believed that KNDy neurons release NKB, which acts on other KNDy neurons via specific receptors to stimulate kisspeptin release (Navarro *et al.*, 2011; Wakabayashi *et al.*, 2010) [but see (Kinsey-Jones and others, 2012)]. Like *KISS1* and its receptor, inactivating mutations of *TAC3/Tac2* (which encodes NKB) or *TACR3* (the gene encoding the NKB receptor), results in pubertal failure (Topaloglu and others, 2008). NKB and kisspeptin are released periodically, and this oscillatory behavior is thought to be determined by a phase-delayed inhibitory feedback of dynorphin on NKB release (Navarro *et al.*, 2011; Wakabayashi *et al.*, 2010). Dynorphin is an opioid peptide that inhibits GnRH secretion (Kinoshita and others, 1982; Navarro and others, 2009; Schulz and others, 1981). Diagrams describing these interactions have been published (d'Anglemont, X *et al.*, 2010; Lehman *et al.*, 2010; Wakabayashi *et al.*, 2010).

The inhibitory transsynaptic circuitry controlling GnRH release involves at least three different neuronal subsets. GABAergic and opiatergic neurons have been known for many years to be central players [reviewed in (Terasawa *et al.*, 2001)]. More recently, evidence has been provided suggesting that RFamide-related peptide (*RFRP*), the mammalian ortholog of the peptide gonadotrophin-inhibiting hormone (GnIH) in birds (Ebling and Luckman, 2008), is a physiological inhibitor of GnRH neurons in mammals (Ducret, Anderson, and Herbison, 2009; Gibson and others, 2008; Tsutsui and others, 2010). As such,

RFRP-containing neurons may be significant components of the restraining mechanism that maintains GnRH secretion in check during reproductive maturation. RFRP neurons may use one or two peptides (RFRP1 and RFRP3) for transsynaptic communication; these peptides are recognized by a high-affinity receptor termed GPR147 or NPFFR1 (Hinuma and others, 2000; Tsutsui *et al.*, 2010), and a low-affinity receptor termed GPR74 or NPFFR2 (Fukusumi, Fujii, and Hinuma, 2006). Because GPR147 is expressed in GnRH neurons (Ducret *et al.*, 2009; Poling and others, 2012), RFRP-containing neurons can directly repress GnRH neuronal function. In contrast to this simplicity, GABAergic neurons can affect GnRH secretion indirectly, via inhibitory effects exerted on neurons connected to the GnRH neuronal network (Ojeda *et al.*, 2006; Terasawa *et al.*, 2001), or directly using excitatory mechanisms set in motion by the activation of $GABA_A$ receptors located on GnRH neurons themselves (DeFazio and others, 2002; Moenter and DeFazio, 2005). Opiatergic neurons appear to exert a pure inhibitory tone to GnRH neurons, but this input is provided by different peptides acting on different receptors (Kordon *et al.*, 1994); as in the case of GABAergic inputs, opiatergic inhibition may be exerted directly on GnRH neurons (Dudas and Merchenthaler, 2006) or indirectly on neurons involved in the stimulatory control of the GnRH neuronal network, such as KNDy neurons of the ARC (Navarro *et al.*, 2009). Additional components of the regulatory system controlling the onset of female puberty include novel molecules required for glutamate release (Choi and others, 2008; Ha and others, 2008) and GnRH neuron excitability (Garcia-Rudaz and others, 2008).

In addition to neurons, the pubertal activation of GnRH secretion involves the participation of glial cells (Ojeda *et al.*, 2010; Ojeda *et al.*, 2008b). Astrocytes and tanycytes (ependymoglial cells lining the ventro-lateral surface of the third ventricle) produce signaling molecules that stimulate GnRH release, and contribute to determining the timing of puberty [reviewed in (Lomniczi *et al.*, 2009; Ojeda *et al.*, 2010)]. Glial cells facilitate GnRH secretion via two complementary mechanisms. One of them involves growth factors of at least four different families [reviewed in (Mahesh, Dhandapani, and Brann, 2006; Prevot, 2002)]. Transforming growth factor-beta (TGFβ) is recognized by cell-membrane receptors endowed with serine-threonine kinase; the epidermal growth factor (EGF) family, basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-I) are recognized by receptors with tyrosine kinase activity. Genetic disruption of erbB (erythroblastosis B) receptors (which recognize the members of the EGF family) delays female sexual development due, at least in part, to impaired erbB ligand-induced glial prostaglandin $E_2$ ($PGE_2$) release (Lomniczi *et al.*, 2009). Preventing the proteolytic processing of erbB ligands in astrocytes delays puberty (Lomniczi and others, 2006) and disrupting astrocytic $PGE_2$ production drastically diminishes the electrophysiological activity of GnRH neurons (Clasadonte and others, 2011).

The second mechanism of glia-to-GnRH neuron communication involves plastic rearrangement in cell adhesiveness provided by at least three different cell-cell communications systems: One involves the sialylated form of the neural cell adhesion molecule NCAM (PSA-NCAM) (Parkash and Kaur, 2005; Perera, Lagenaur, and Plant, 1993). Another requires the adhesion molecule Synaptic Cell Adhesion Molecule 1 (SynCAM1) (Sandau and others, 2011a; Sandau and others, 2011b), and a third one is based on the interaction of neuronal contactin with glial Receptor-like Protein Tyrosine Phosphatase-β (RPTPβ) (Parent and others, 2007). All three systems use adhesive proteins containing intracellular signaling domains, suggesting that the interaction of glial cells with GnRH neurons not only involve secreted bioactive molecules (growth factors, prostaglandins, etc.), but also intracellular signaling mechanisms set in motion by adhesive molecules [reviewed in (Lomniczi *et al.*, 2009)]. A more comprehensive coverage of the mechanisms controlling the onset of puberty can be found in earlier reviews (Ojeda *et al.*,

2006; Ojeda and Urbanski, 1994; Tena-Sempere, 2006; Terasawa *et al.*, 2001), and in several chapters of this book (Tena-Sempere, Megan Hagenauer/Terri Lee, Dennis Styne).

## The neuroendocrine control of puberty involves many genes of different functions

The demonstration in 2003 by two independent groups that the lack of functional GPR54 receptors results in pubertal failure (de Roux *et al.*, 2003; Seminara *et al.*, 2003) energized the field of puberty to an unprecedented extent, leading to a flurry of publications suggesting a vital role of kisspeptin for a diversity of reproductive functions both at central and peripheral levels. It would appear intuitively evident, however, that the kisspeptin system does not work in isolation. Maintaining reproductive function is such an essential requirement for the preservation of the species that the robustness of the system might be seriously compromised if it did not have built-in safeguard mechanisms to ensure functional continuity in the event of loss of a component. Accordingly, the neuroendocrine regulatory complex within which *Kiss1* operates would be expected to follow the same general principles governing other biological networks. Though controlled by a small number of genes, these networks are robust (that is, they have a significant degree of internal redundancy) and are endowed with a high degree of error tolerance (Basso and others, 2005). This general feature appears to be valid for the control of GnRH secretion, because the early removal of kisspeptin neurons appears to be followed by the activation of compensatory mechanisms (Mayer and Boehm, 2011) that reestablish normal reproductive function. There are also compensatory mechanisms that allow reproductive capacity to be sustained in the absence of *Tac3R* (Yang and others, 2012), indicating that in each case loss of a pivotal element of the network is followed by activation of alternative pathways. A note of caution needs to be introduced here because ablation of kisspeptin neurons did not eliminate all of these neurons, which raises the possibility that only a few kisspeptin neurons are required for normal function.

These compensatory pathways may require some of the many genes implicated in the neuroendocrine control of puberty (Eaves and others, 2004; Gajdos and others, 2008; Krewson and others, 2004; Ojeda and others, 2006; Seminara and Crowley, Jr., 2001). Although monogenic mutations affecting genes such as *GNRHR* (Bedecarrats and Kaiser, 2007), *GPR54* (de Roux *et al.*, 2003; Seminara *et al.*, 2003), *KiSS1* (Lapatto *et al.*, 2007; Topaloglu *et al.*, 2012), *TAC3* and *TACR3* (Topaloglu *et al.*, 2008), result in absence of pubertal development, the combined fraction of individuals affected by these mutations does not surpass 5% of the total population affected by pubertal disorders. The contribution of additional genes is suggested by the results of genome-wide association studies demonstrating that sequence variations in at least 30 genes are associated with an early age at menarche (He and others, 2009; Ong and others, 2009; Perry and others, 2009; Sulem and others, 2009). Prominent among these genes are the post-transcriptional repressor *LIN28b* (He *et al.*, 2009; Ong *et al.*, 2009; Perry *et al.*, 2009) and several genes encoding proteins associated with body mass index, energy homeostasis and hormone regulation (Elks and others, 2010). Also of interest is the unexpected association of two Zinc-finger (ZNF) genes, ZNF462 (Perry *et al.*, 2009) and ZNF483 (Elks *et al.*, 2010) with the age at menarche. ZNFs constitute a large family of transcriptional repressors (Filion and others, 2006; Shannon and others, 1996; Urrutia, 2003; Vogel and others, 2006), some of which can bind to both DNA and RNA (Burdach and others, 2012) to inhibit gene expression. In addition to these findings, earlier studies from our laboratory identified several transcriptional regulators of puberty, such as the POU-domain gene *Oct2* (Ojeda and others, 1999), the homeodomain gene *Ttf1/Nkx2.1* (Mastronardi and others, 2006), and a novel gene (Rampazzo and others, 2000), which we termed *Eap1* (**E**nhanced **A**t **P**uberty1) (Heger and others, 2007).

The above considerations make it clear that there are numerous neuroendocrine genes that are necessary for the onset of puberty, but that there are redundancies in the system. Therefore, it is import to focus on the upper echelon genes regulating the expression or repression of the neuroendocrine axis. Accordingly, this review will focus on the upstream transcriptional regulators of the pubertal process. We will first provide the reader with some basic information about the approaches one may use to identify, interpret and integrate the information derived from high throughput data into a biologically testable model.

## General Structure of a Genetic Network

Genetic networks are composed of individual elements that interact with each other (Klipp and others, 2005). Some of these elements act as “portals” receiving and processing information from the environment; others, function mostly within the network. All networks contain central “hubs”, which are strongly interconnected and direct the flow of information throughout the entire network. Subordinate “nodes”, located at various hierarchical positions (and within different intra– and inter-cellular levels), respond to commands emanating from the central hubs, and relayed to them either directly or via first and second “neighbors”. This organization allows for the regulation of a complex system using relatively few genes, since the complexity of the system comes from the connectivity and not so much from the number of genes or hubs. Signal propagation within the network may be both fast, but evanescent, and slow, but stable (Luscombe and others, 2004). Because the network’s nodes are strongly interconnected and this connectivity does not increase (i.e. it is not “scaled up”) as the complexity of the network increases, genetic networks are considered to be scale-free (Basso *et al.*, 2005). In addition, they are hierarchical, because highly connected nodes exhibit preferential interactions among themselves (Klipp *et al.*, 2005). Due to this architectural arrangement, networks contain “upper echelon” hubs that are highly interconnected, and a multitude of peripheral nodes that become increasing less connected as they move away from the major hubs (Fig. 1). This loss of connectivity is not surprising because the size (and complexity) of a network can increase markedly as it expands from its core (see for instance (Basso *et al.*, 2005; Carro and others, 2010; Luscombe *et al.*, 2004). Groups of genes exhibiting a high overlap of connected nodes are considered as “modules” within a network. It is important to also consider that the interactions between transcriptional nodes may vary in response to different stimuli, suggesting that rewiring is a biological feature of network function (Luscombe *et al.*, 2004). An additional feature worth mentioning is that the “edges” of the network (i.e., the connectivity) can consist of multiple types of interactions (co-expression, physical contact, transcriptional control, etc.) all of which complement and overlay one another.

## Tools for construction and visualization of biological networks

An initial step in the identification of biological networks is the use of high throughput approaches (such as DNA arrays and proteomics). A late step involves perturbation of the system (by either overexpressing or silencing key components of the presumed network) (Davidson and others, 2002). Below, we describe our workflow, which is representative of current methodologies in biological network construction. A main computational framework that can be employed to identify the main modules, which define the high-level architecture of the network, is Weighted Gene Co-expression Network Analysis (WGCNA) (Zhang and Horvath, 2005). This framework allows the investigator to (i) construct gene co-expression networks, (ii) identify network modules and their relationships, (iii) relate the identified modules and module interactions to external information, such as functional enrichment and protein-protein interaction, and (iv) find key drivers in modules of interest with respect to the central hypothesis in order to identify new targets for experimental validation via RNAi silencing/gene overexpression (see for instance (Carro *et al.*, 2010; Iancu and others, 2010;

Ponomarev and others, 2012)). Alternative strategies for the construction of biological networks have been employed by other investigators (for instance see (Carro *et al.*, 2010; Ideker and others, 2001; Longabaugh, Davidson, and Bolouri, 2005; Ueda and others, 2005).

To aid our analysis we have developed an extended version of the WGCNA R package (Langfelder and Horvath, 2008) (http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/) (augmented with local connectivity analysis and alternative topological overlap measures). Using this tool, we first compute the Pearson correlation coefficient for all pairs of genes in an expression data set and produce a matrix of pairwise correlations. We then transform the correlation matrix into an adjacency matrix using a power function that computes the connection strength between a pair of probes by raising the correlation value to a power determined by the scale-free topology criterion (Zhang *et al.*, 2005). It may also be possible to employ the Maximal Information-based Nonparametic Exploration (MINE) association metric (Reshef and others, 2011) (http://www.exploredata.net/) as an alternative or supplement to the Pearson correlation. Because MINE can detect nonlinear associations between variables, it can capture biologically relevant relationships between genes that the Pearson correlation may not detect.

## Module identification

With the connection strengths determined in an adjacency matrix, the next step is to identify modules in the network. The degree to which a pair of genes share neighbors is measured by the 'topological overlap' metric (Ravasz and others, 2002; Zhang *et al.*, 2005), which accounts for the number of genes connected to both genes normalized by the total number of connections. We have extended the topological overlap metric by using graphlet counts (Przulj, 2007), a technique for measuring refined local connectivity. We have developed a publicly available CytoScape plug-in, GraphletCounter (Whelan and Sonmez, 2012) that computes the number of subnetworks (up to 5 nodes) to which a node is connected, producing a connectivity feature vector. We use inner products of the connectivity vectors as an alternative topological metric. Using a topological overlap measure as opposed to thresholding the raw adjacency values reduces the spurious connection strengths and results in a more robust method for estimating putative modules. With the topological overlap matrix, we identify the network modules as branches of the clustering tree resulting from the application of the "dynamic tree cutting algorithm" (Langfelder, Zhang, and Horvath, 2008), which takes advantage of the internal structure of the dendrogram in cutting the branches and identifying modules.

## Functional enrichment

Each module's gene make-up is tested for GO category enrichment (Ashburner and others, 2000) using the GOstats R package (Falcon and Gentleman, 2007) (http://www.bioconductor.org/packages/2.10/bioc/html/GOstats.html). To remove biases due to the nested structure of the GO terms, a graph decorrelation procedure (Alexa, Rahnenfuhrer, and Lengauer, 2006) is employed. The resulting p-values are adjusted for multiple hypotheses testing using False Discovery Rate (FDR) and transformed into q-values (Storey, 2003), as well as by the Bonferroni correction.

## Static models of biological networks

A gene network for the control of puberty can be constructed by combining a large variety of functional association data in the meta-network framework GeneMANIA (Warde-Farley and others, 2010), thus allowing the prediction of the function of modules and/or gene sets. GeneMANIA finds interactions and complementary genes that are related to a set of input genes. For the control of puberty, the input sets may be generated using (i) existing gene lists from preliminary data explained in detail in the preceding sections, (ii) genes that

display a specified temporal expression pattern in microarray experiments, or (iii) a combination of both. Association data available for forming complementary network edges include protein and genetic interactions, pathways, co-expression, co-localization and protein domain similarity. The basic idea is to find new members of a pathway or complex, find additional genes that may have been missed or augment the network with new genes with a specific function.

GeneMANIA accomplishes this goal by searching for related genes in many large, publicly available biological datasets, including protein-protein, protein-DNA and genetic interactions, pathways, reactions, gene and protein expression data, protein domains and phenotypic screening profiles. Main datasets such as BioGRID, PathwayCommons, Pfam are part of the search as well as datasets from a wealth of datasets from recent papers. The data are available for human, mouse and rat, and the static networks built for the three organisms can be compared, complemented, and novel orthologs identified.

## Proteome interactions: transcriptome co-expression and transcription factor (TF) binding sites

To perform this analysis, the gene network co-expression patterns are compared with a manually compiled protein-protein interactions (PPI) database retrieved from the Human Protein Reference Database (HPRD) (Keshava Prasad and others, 2009; Peri and others, 2003). Using Entrez IDs, one can select the network genes that are also present in the list of HPRD gene products. The network genes with PPI interactions can then be selected and used to compute the average topological overlap. Genomic upstream DNA sequences are analyzed for TF binding sites using TRANSFAC (http://www.biobase.de), and the results are incorporated into the network model. We are currently attempting to incorporate epigenetics information to the analysis of predicted transcription factor binding sites by applying a technique we recently developed (Wright and others, 2011) to estimate occupancy of predicted sites based on nearby epigenetic markers.

## Dynamic developmental models via BioTapestry

Because network models can be generated to include multiple developmental time point variations in gene expression, a model of gene action over time can be created. The resulting networks can be visualized using the CytoScape software (Shannon and others, 2003) (http://www.cytoscape.org), which allows for interactive exploration of interactions in a network. The CytoScape network can be overlaid with expression data to help identify active pathways in the network. To facilitate this process one can explore the KEGG pathway database for all known pathways containing the highest number of genes from the set of interest. The resulting CytoScape models for each time point can then be saved in a Systems Biology Markup Language (SBML) format (Finney and Hucka, 2003) allowing it to be viewed, analyzed, and refined using software packages, such as the Systems Biology Workbench (SBW) (Sauro and others, 2003) (http://sbw.sourceforge.net). SBML also allows the investigator to share models easily with other research groups. Incorporating temporal and spatial characteristics of gene expressions in the model can be achieved using the BioTapestry software (Longabaugh *et al.*, 2005) (http://www.biotapestry.org). The BioTapestry model can be used to capture the regulation of gene transcription network and animate observed changes in gene expression patterns over time, such as those that occur during pre- and peripubertal development. The different components of a pipeline that can be used for the computational analysis and representation of biological networks are shown in Fig. 2.

## A gene network controlling puberty

An issue raised by these observations is whether these genes interact with other elements of the neuroendocrine brain to form a biological network with functional significance. Using high-throughput, genomic and proteomic approaches (Choi *et al.*, 2008; Ha *et al.*, 2008; Heger *et al.*, 2007; Lee and others, 2001; Mastronardi *et al.*, 2006; Ojeda *et al.*, 1999; Ojeda *et al.*, 2006; Parent and others, 2008; Roth and others, 2006), in combination with some of the tools described above, we obtained an initial glimpse into the general structure and hierarchical arrangement of one of these networks (Ojeda and others, 2010; Ojeda *et al.*, 2006; Roth and others, 2007; Roth *et al.*, 2006). We used the medial basal hypothalamus of both female rats and female rhesus monkeys for DNA array analysis. The rats were euthanized at three different stages, juvenile (25-days of age), early puberty (30–35 days of age) and on the day of the first proestrus (32–37 days of age). These stages were defined according to established criteria (Ojeda *et al.*, 2006). Accumulation of uterine fluid (an indication of estradiol secretion) begins during early puberty and becomes maximal on the day of the first proestrus. However, vaginal opening does not usually occur until the next day, which is the day of the first ovulation. The monkeys were also euthanized at three developmental phases: juvenile (8.9 month–1.8 year-old), early pubertal (2–3-year old) and midpubertal (3–4 year-old). LH levels are low in juvenile animals and begin to increase during the early pubertal period to become distinctly elevated by midpuberty. Analysis of the array data followed by quantitative PCR verification revealed that expression of several genes earlier identified as involved in tumor suppression/tumor formation increases in the hypothalamus at the time of puberty in both rats and monkeys (Roth *et al.*, 2007). *Cis*-regulatory analysis of shared binding sites predicted the existence of five central hubs (*Cutl1/Cdp/Cux1, Maf, p53, Yy1, and Usf2*) controlling the network at the transcriptional level (Roth *et al.*, 2007). The model also predicted the existence of many subordinate genes, including *KiSS1* (previously known as suppressor of metastasis) (Kotani and others, 2001; Steeg and others, 2003) and *SynCAM1* (previously known as tumor suppressor of lung cancer, *TSLC1*) (Kuramochi and others, 2001; Watabe and others, 2003) (Fig. 3). While *KiSS1* is essential for the occurrence of puberty in mice and humans, we now know that *SynCAM1* plays a pivotal role in the developmental control of glia-GnRH neuron adhesive communication (Sandau *et al.*, 2011b), and in the mechanism by which astrocytes facilitate GnRH release and control normal female reproductive function (Ojeda, Lomniczi, and Sandau, 2008a; Sandau *et al.*, 2011a).

Interestingly, *in silico* analysis of the network predicted a connection between these central hubs and *Oct2, Ttf1,* and *Eap1,* the three genes implicated by other studies as upstream transcriptional regulators of the pubertal process (Heger *et al.*, 2007; Mastronardi *et al.*, 2006) (Fig. 3). Immunohistochemistry and *in situ* hybridization analysis demonstrated that these genes, as well as other subordinate nodes of the network, are expressed in neuronal and/or glial subsets involved in the control of GnRH secretion, including GnRH neurons themselves (Heger *et al.*, 2007; Mastronardi *et al.*, 2006; Ojeda *et al.*, 2008b; Roth *et al.*, 2007). A recent assessment of some of these *in silico* predictions (Mueller and others, 2011) showed that that *KISS1* expression is not only controlled by TTF1 and EAP1, but also by CUTL1/CUX1 and YY1, two putative central hubs of the TRG network. While TTF1 and the long form of CUX1 (known as p200) are trans-activational, a short form of CUTL1 (p110) and YY1, and to some extent, EAP1 are repressive (Mueller *et al.*, 2011). Using ChIP assays, we observed that all four of these transcription factors are recruited to the *KiSS1* promoter; immunohistofluorescence studies showed that they are present in kisspeptin neurons *in vivo* (Mueller *et al.*, 2011). We further observed that *EAP1* gene expression is also under dual transcriptional regulation imposed by TTF1 (trans-activation) and YY1 and CUX1 (repression), and that EAP1 itself appears to control its own expression via a negative auto-feedback loop mechanism (Mueller and others, 2012). Consistent with

the general features of a transcriptional regulatory network, TTF1, YY1 and CUX1 associate with the EAP1 promoter region and are expressed in hypothalamic EAP1 neurons, indicating that they are physiologically involved in the control of *EAP1* gene expression. Although we initially considered EAP1 and TTF1 as non-TRGs molecules (Ojeda *et al.*, 2010), it has been recently shown that both are implicated in the biology of cancer (Winslow and others, 2011; Yeung and others, 2011). Based on this information, we now believe that these genes can be considered as part of a distinct module within the TRG network (Fig. 3).

To assess the importance of TTF1 and EAP1 in the control of reproductive function we used loss-of function approaches. Conditional deletion of the *Ttf1* gene in mice resulted in delayed puberty and disrupted estrous cyclicity (Mastronardi *et al.*, 2006). Microinjection of lentiviral particles carrying a siRNA against *Eap1* mRNA into the preoptic area of juvenile female rats, also delayed puberty and disrupted estrous cyclicity (Heger *et al.*, 2007). Similar injections into the medial basal hypothalamus of rhesus monkeys obliterated menstrual cyclicity (Dissen and others, 2012) indicating that *EAP1* is crucial for the ARC to maintain menstrual cyclicity in higher primates. This role is supported by the recent finding that a single nucleotide polymorphism in the 5′-flanking region of the *EAP1* gene is associated with reduced *EAP1* transcription and oligomenorrhea/amenorrhea in nonhuman primates (Lomniczi and others, 2012a).

The draft model of this TRG network is imperfect in many different ways. Firstly, although based on interactions taking place at different stages of pubertal development, it essentially depicts a static view of these interactions. Secondly, it does not identify the cell type (neurons or glia) where these interactions may be occurring. Lastly, it does not appropriately considered the homeostatic process of post-translational processing that - required for formation of biological active peptides (Artenstein and Opal, 2011) – may be essential for both the short and the long-term activity of the regulatory system.

## The potential involvement of additional transcriptional regulatory networks in the control of puberty

Although some central TRG nodes can function as transcriptional repressors (*YY1, EAP1, CUX1*), there are at least four additional transcriptional and post-transcriptional repressors that may contribute to controlling the timing of puberty. One of them is *LIN28b,* which encodes an RNA binding protein that inhibits the maturation of let7 miRNAs (Hagan, Piskounova, and Gregory, 2009; Heo and others, 2009; Lehrbach and others, 2009), a family of microRNAs with tumor suppressor activity (Chang and others, 2009). The potential contribution of *LIN28b* to the regulation of puberty was suggested by the finding that a single nucleotide polymorphism near the *LIN28B* gene in human chromosome 6(q21) is associated with earlier puberty and shorter stature in girls (Ong *et al.*, 2009; Perry *et al.*, 2009; Sulem *et al.*, 2009).

A broad mechanism of transcriptional repression imposed by members of the ZNF superfamily (Filion *et al.*, 2006; Shannon *et al.*, 1996; Urrutia, 2003; Vogel *et al.*, 2006) may be another regulatory component of the pubertal process. This notion, still in its infancy, derives from high-throughput studies of the agonadal male monkey hypothalamus showing that a reduced expression of several ZNFs accompanies the initiation of the pubertal process in primates (Matagne and others, 2009). Finally, we recently found that members of two additional families of transcriptional repressors are differentially expressed in the peripubertal female rat hypothalamus. One of these families is composed of POZ-ZF proteins, which control a variety of developmental processes via transcriptional repression. Alteration of their function leads to tumorigenesis and developmental disorders (Kelly and Daniel, 2006). We found that expression of three genes belonging to a subset of the POZ-ZF

gene family, known as the *POK* subfamily (Costoya, 2007), increases in the hypothalamus of female rats at puberty (unpublished observations), suggesting that these genes may function as repressors of other downstream repressors of puberty. In contrast to this pattern of expression, expression of two key members of the PcG (Polycomb) complex of gene silencers (Cbx7 and Eed) *decreases* in the hypothalamus at puberty (Lomniczi, Loche, and Ojeda, 2010), suggesting that – as described below – these genes might prevent the untimely, earlier initiation of puberty by inhibiting the expression of downstream genes whose activation is ultimately required for puberty to occur. The PcG silencing complex is known for its ability to impose gene silencing at various developmental stages (Kohler and Villar, 2008; Schwartz and Pirrotta, 2007; Simon and Kingston, 2009).

We currently believe that POK and PcG genes may operate within the TRG network itself (Fig. 4), because a) both have tumor suppressor activity (Classen and others, 2009; Kelly *et al.*, 2006; Martinez and others, 2009), b) PcGs are recruited to the promoter of a puberty-activating gene (*Kiss1)* and restrain *Kiss1* expression both *in vivo* and *in vitro* (Lomniczi and others, 2012b), and c) POK proteins are, in turn, recruited to PcG gene promoters to repress their activity (unpublished data). We speculate that LIN28b and ZNFs, form associated subnetworks; however, the boundaries of presumed ZNF-driven network may have to be redefined, because some ZNFs display features that makes them structurally similar to the POK family (Costoya, 2007). Because LIN28b delays development via post-transcriptional suppression of microRNA maturation (Hagan *et al.*, 2009; Heo *et al.*, 2009; Lehrbach *et al.*, 2009) instead of transcriptional silencing of target genes, we infer that *Lin28b* is a central hub of an associated repressive network (Fig. 4). Needless to say, more work is required before all these genes can be accurately assigned to specific modules within the topological architecture of each network (Fig. 4).

## The contribution of epigenetics

Despite the fact that most, if not all, of the aforementioned genes are subjected to epigenetic regulation (Ball and others, 2009; Guenther and others, 2007; Koch and others, 2007; Miao and Natarajan, 2005; Mikkelsen and others, 2007; Weber and others, 2005; Weber and others, 2007) (see also http://www.epigenome.org/, http://epigenome.usc.edu/ and http://epigenomegateway.wustl.edu/), it is only recently that the potential contribution of epigenetics to the regulation of puberty has been addressed. Although not dealing with puberty itself, two recent reports relevant to the subject need to be mentioned here. One of them demonstrated that the GnRH gene itself is subjected to epigenetic regulation by showing that methylation of the GnRH promoter decreases as GnRH expression increase during in vitro fetal maturation of primate GnRH neurons (Kurian, Keen, and Terasawa, 2010). The other demonstrated that sex differences in *Kiss1* expression in the AVPV of mice are related to differences in the degree of *Kiss1* promoter methylation between males and females (Semaan and others, 2012). Using a combination of DNA microarrays, genome-wide DNA methylation arrays, ChIP and gene expression analyses we discovered that an epigenetic mechanism of transcriptional repression operating in the hypothalamus plays a significant role in timing female puberty (Lomniczi *et al.*, 2012b). Our results identified the PcG of transcriptional silencers (Kohler *et al.*, 2008; Schwartz *et al.*, 2007; Simon *et al.*, 2009) as a major contributor to this repressive mechanism, and implicated two PcG genes (Cbx7 and Eed) as core components of the PcG complex operating in the ARC of the prepubertal medial basal hypothalamus.

Since kisspeptin and NKB work coordinately within a single cell type to stimulate GnRH secretion, their encoding genes (*Kiss1* and *Tac2*) can be considered as components of a unique class of puberty-activating genes. Using the *Kiss1* gene as a prototype of this class, we showed that both the *Eed* and *Cbx7* genes are expressed in kisspeptin neurons of the

ARC, and that the CBX7 and EED proteins are associated with the 5′ flanking region of the *Kiss1* gene. At the end of juvenile development, coinciding with the pubertal augmentation of pulsatile LH release (Urbanski and Ojeda, 1985), the *Eed* and *Cbx7* promoters become more methylated and the expression of both *Eed* and *Cbx7* decreases. As PcG proteins are evicted from the *Kiss1* promoter, association of histone marks involved in gene activation (H3K9,14ac, and H3K4me3) is enhanced *and Kiss1* expression increases. When the pubertal decline in hypothalamic EED and CBX7 expression was prevented by overexpressing these proteins in the ARC of early juvenile rats, the number of immunoreactive KNDy neurons in the ARC was reduced, puberty was delayed, estrous cyclicity was disrupted, and importantly, fertility was severely compromised (Lomniczi *et al.*, 2012b). These results suggest that similar or complementary mechanisms of epigenetic regulation may coordinate and dynamically integrate signal propagation within gene networks involved in the neuroendocrine control of puberty (Fig. 4). PcG, POK and ZNF proteins illustrate this feature; while PcG proteins repress gene transcription by imposing histone modifications associated with gene silencing (Sawarkar and Paro, 2010; Simon *et al.*, 2009), POK proteins exert their repressive effect by recruiting co-repressors and histone deacetylases (Costoya, 2007; Koh and others, 2009; Lemercier and others, 2002). Similarly, ZNFs impose gene silencing by recruiting co-repressors, which in turn recruit histone methyltransferases needed for the synthesis of repressive histone marks (Frietze and others, 2010).

## Conclusions and Perspectives

The aforementioned observations are consistent with the view that the onset of puberty depends on the contribution of more than one gene. Puberty also appears to be controlled at the transcriptional/post-transcriptional level by discrete groups of genes. We envision the participation of “activators” that move the process along by promoting key developmental events, and “repressors” that prevent the untimely activation of activating genes (Fig. 3 and 4). These elements may be organized in at least three functionally connected sub-networks, one centered about *LIN28b*, another composed of *TRGs* and a third one containing *ZNFs* (Fig. 4). It would be reasonable to assume that – even though of different compositions – these regulatory systems operate within both the neuronal and glial populations involved in controlling the onset of puberty. The results thus far obtained suggest that a two-tiered hierarchy of repressive nodes provides “hard-wiring” to the networks. One of the repressive layers, composed of transcriptional and post-transcriptional “repressors” (such as *PcG* genes and *Lin28b*) may directly prevent the premature activation of puberty-inducing genes (such as *Kiss1, Tac3, Ttf1*, etc). Another layer, located at a higher hierarchical level in the network is formed by repressors such as *POK/ZNF* genes. Because these genes may modulate the expression of repressors (Fig. 4), they can be considered as “repressors of repressors”. Both layers may directly regulate subordinate genes of the network involved in the stimulatory (Kisspeptin, glutamatergic, glial) and inhibitory (GABA, opioid, RFRP3) control of GnRH secretion. In our view, an essential feature of this model is the presence of a dynamic flow of epigenetic information (Lomniczi *et al.*, 2010; Ojeda and Lomniczi, 2010), which may provide a granular level of coordination and transcriptional plasticity to the sub-networks. We hope that the concepts proposed here provide a framework for future investigation in the area of mammalian puberty.

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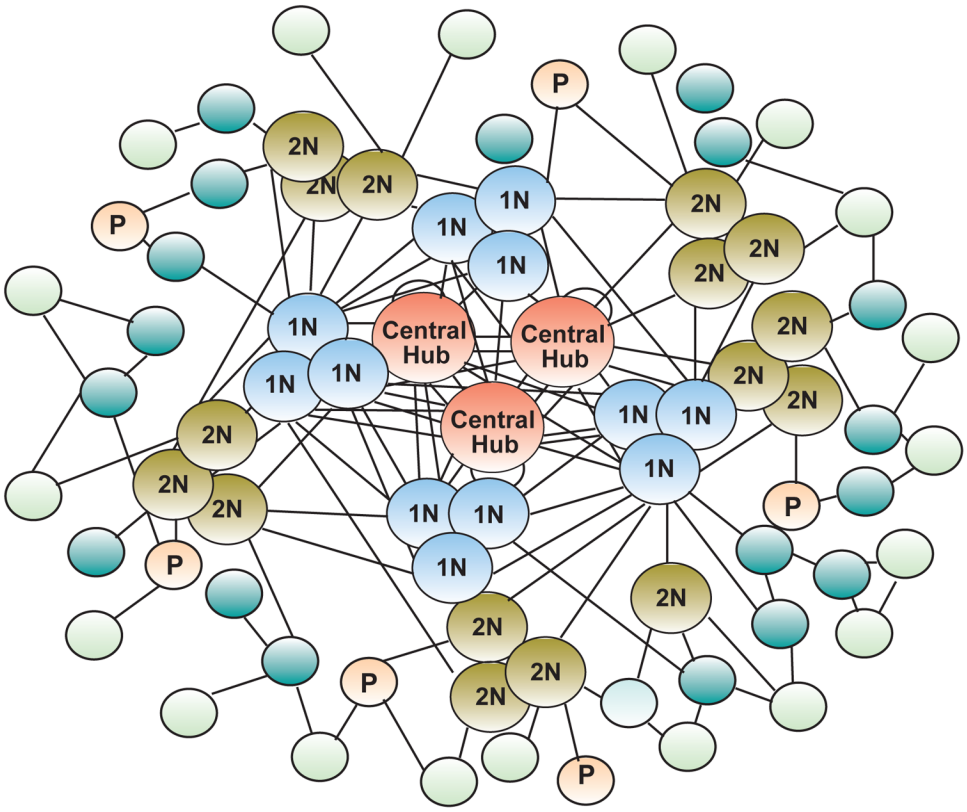


**Figure 1.**

The general organization of a biological network. The individual elements composing the network interact with each other. Some of these elements (P, portals) receive information from the environment; others, function mostly within the network. The central “hubs” or “nodes” are strongly interconnected and direct the flow of information throughout the entire network. Subordinate nodes respond to commands emanating from the central hubs; this information can be relayed to them either directly or via first (1N) and (2N) second “neighbors”. The architectural arrangement of the network is such that they contain “upper echelon” nodes that are highly interconnected, and a multitude of peripheral nodes that become increasing less connected as they move away from the major hubs. The arrangement of first and second nodes in clusters of three illustrates the concept that the network contains “modules”, i.e. sub-groups of genes with highly related functions. Depending on the data type, interactions between nodes (termed “edges”) may represent co-expression, physical association, transcriptional control, or other types of gene or gene product interactions. The nodes are colored according to their relative position within the network: orange = central nodes; light blue = first neighbors; light brown = second neighbors; light orange = portal nodes; dark blue = subordinate nodes located sub-peripherally; light green = most peripherally located nodes.

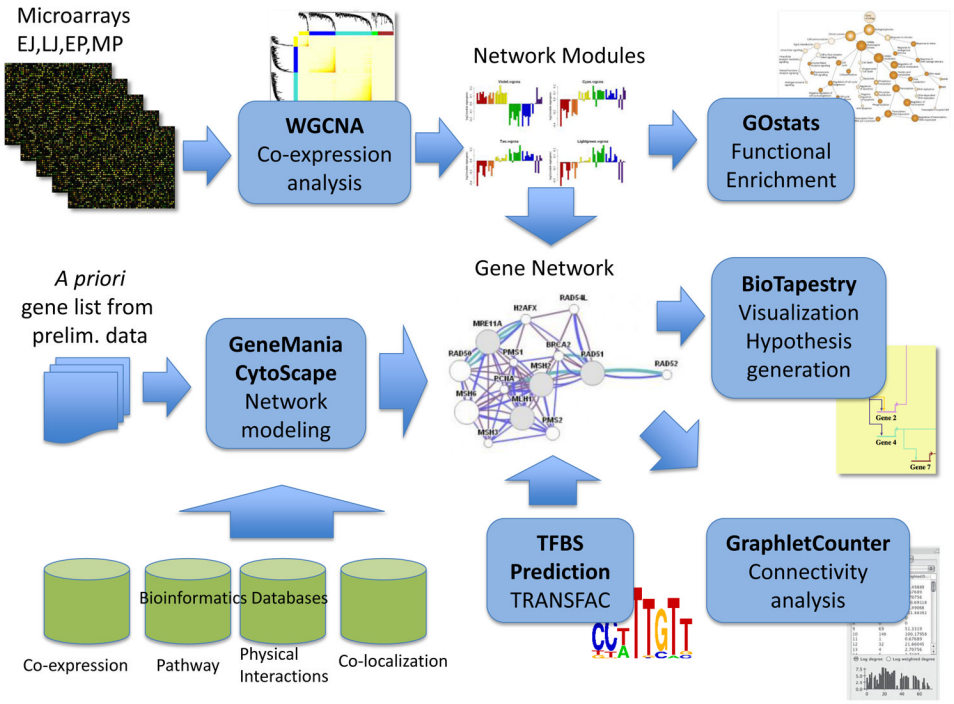


**Figure 2.**

Bioinformatics pipeline and network construction framework. High-throughput experimental data (such as expression) are analyzed both (i) *de novo*, via Weighted Gene Co-expression Network Analysis (WGCNA), resulting in network modules and their associated enrichment in Gene Ontology categories, such as biological process, molecular function and subcellular localization, and (ii) by building gene networks based on genes identified in preliminary experiments and/or demonstrating a certain temporal expression profile, drawing from a rich battery of datasets via GeneMANIA, a network construction framework that works within CytoScape. Prediction of functional elements such as transcription factor binding sites further elucidates the gene network through the use of motifs in TRANSFAC and the latest ChIP-seq data in ENCODE. The resulting network is visualized and overlaid with expression data in either CytoScape or BioTapestry, allowing the generation of hypotheses for experimental validation (for instance, via RNAi-mediated silencing). The network can also be analyzed for connectivity using GraphletCounter, a publicly available CytoScape plug-in that computes the number of subnetworks (up to 5 nodes) to which a node is connected. EJ = early juvenile period; LJ = late juvenile period; EP = early proestrus, the phase of puberty in the rat when uterine fluid begin to accumulate indicating increased estrogen secretion; MP = midpuberty, the pubertal phase that correspond to the day of the first preovulatory surge of gonadotropins in the rat; A =adulthood; TFBS = transcription factor binding sites.

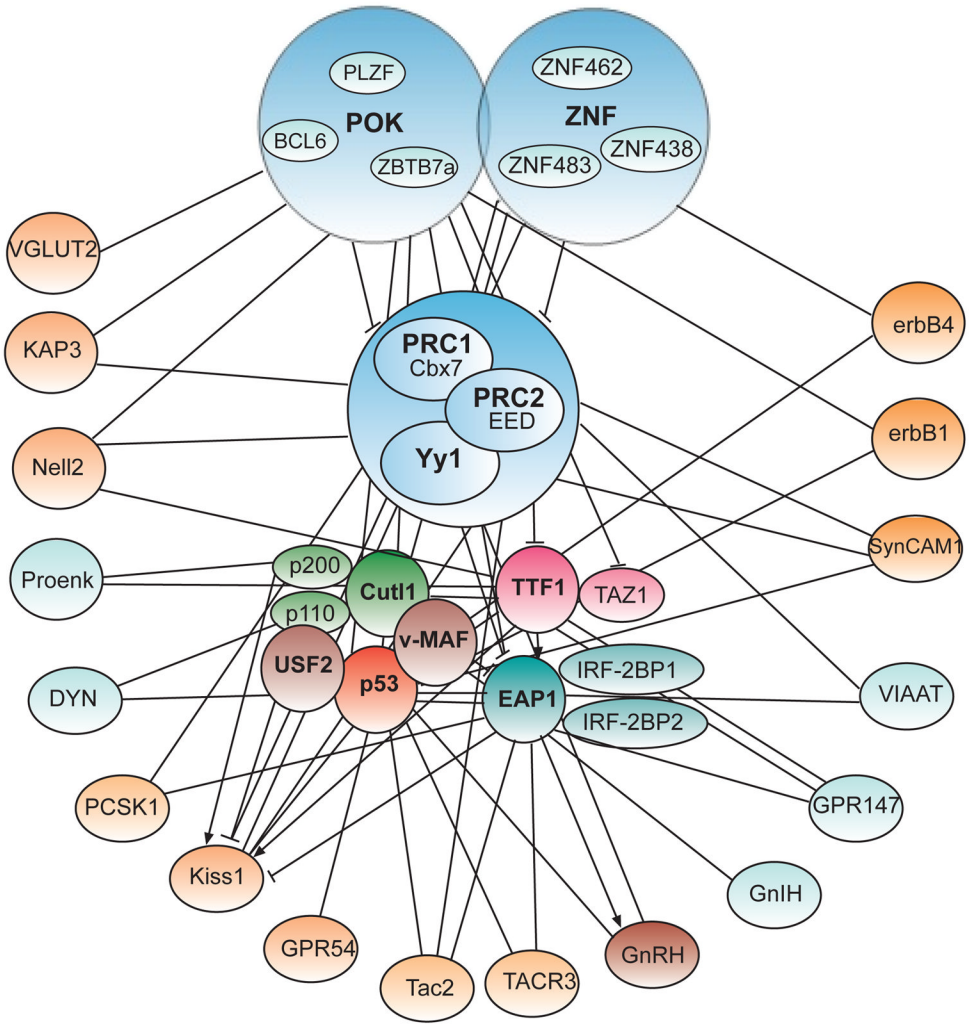


**Figure 3.**

Key features of the TRG network. Two modules composed of different types of transcriptional regulators are postulated to be strongly interconnected and to directly control the activity of peripheral nodes via either trans-activational or repressive mechanisms (← = trans-activation; ⊥ = transcriptional repression). In one of these modules, *CUTL1 and p53*, function as central hubs; in the other, this role is played by *TTF1* and *EAP1*. Both modules regulate the expression of a host of subordinate genes, including puberty activating genes (e.g., *KISS1*) and genes involved in the inhibitory control of puberty (e.g. opioid peptides, RFRP3/GnIH). The model predicts that the TRG network is subjected to a two-tiered hierarchy of repressive control provided by two modules. One consisting of POK/ZNF genes, would serve are *repressor of repressors*. The other repressive module is composed of PcG genes. YY1, which in our initial model was considered to be a central TRG node (Roth *et al.*, 2007), can now be assigned to the PcG complex. According to the current model, POK/ZNF proteins repress PcG genes, and in turn PcG proteins repress downstream puberty activating genes. POK/ZNF proteins may also suppress directly downstream genes. Large blue modules = “upstream” transcriptional repressors consisting of three different families of transcription factors; smaller centrally clustered modules of different colors = secondary central modules composed of genetically unrelated genes; peripheral light blue nodes = genes considered to be inhibitory to the pubertal process; peripheral orange nodes = genes

considered to be activators of puberty. Lines ending without arrows or blunt ends denote that the nodes are predicted to be connected by in silico analysis (for instance, the presence of transcription factor binding sites in the downstream target gene), but experimental evidence is lacking.

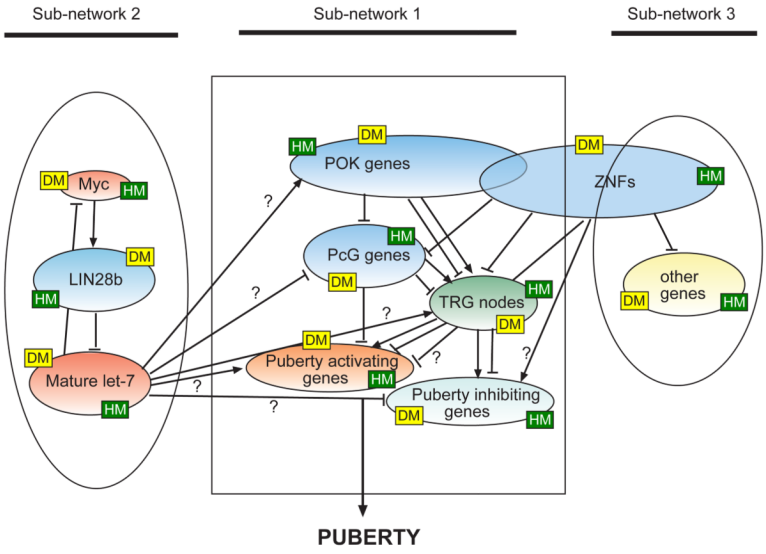


**Figure 4. General organization of hypothetical transcriptional regulatory networks controlling the onset of female puberty**

This draft model predicts the existence of three functionally connected sub-networks. One of them uses TRGs as central hubs; another uses *LIN28b*, and a third, less well-defined, is formed by ZNF genes. The latter may overlap considerably with the POK/ZNF module of the TRG network, because several POK genes also contain a kr ppel C2H2 zinc-finger domain core that is characteristic of many ZNFs. Intra and inter sub-networks information is postulated to be dynamically coordinated by epigenetic mechanisms. According to existing information derived from a vast body of literature, including studies performed in our laboratory, these mechanisms involve changes in DNA methylation (DM) and changes in association of modified histones (HM) to gene promoters. The model predicts that the transcriptional inhibition of puberty activating genes is lifted at or before puberty, and replaced by increased trans-activation of gene expression. Simultaneously, puberty-inhibiting genes may be either repressed or experience a reduction of activating inputs. ⟵ = stimulation; ⊥ = Blue denotes transcriptional/post-transcriptional repressors; light red denotes activators; darker green = central nodes with either trans-activating or repressive activity; lighter green = subordinate genes involved in the inhibitory control of puberty, but that are not transcription factors; yellow = other, not yet identified genes.