Transcription factors regulating neuroendocrine development, function and oncogenesis

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Abstract

Transcriptional regulation contributes to the hierarchy of processes that ensure proteins are expressed in the correct cells at appropriate times. Such exquisite control is critical for neurohormones, which are induced in response to specific physiological signals, and for which inappropriate expression has pathological consequences. The basal transcription of genes is modulated by the sequence-specific binding of transcription factors (TFs) to enhancer or repressor elements within the gene control regions. These TFs are modular proteins, which use specialized DNA-binding domains to interact at the correct DNA sequence motifs. Here they form scaffolds that recruit regulatory co-factors to modify surrounding the chromatin. The nature of these co-factors determines whether a TF functions as an activator or a repressor of transcription (Figure 1). Here we consider classes of TFs that regulate the expression of neuropeptides, drive the development of neuroendocrine tissues, or define neuroendocrine cancers. Genome-wide studies are now beginning to reveal the extent and diversity of the binding motifs for individual TFs. Many TFs regulate transcription of messenger RNAs and also non-coding RNAs, which themselves exert transcriptional or post-transcriptional regulation of gene expression. The regulation of TFs by alternative splicing, post-translational modifications, protein-protein interactions and subcellular re-localization also diversifies their function (Figure 1). TFs often contribute to cascades of transcriptional regulators, or work in feedback loops. Thus transcriptional regulation is complex, co-operative and dynamic, relying on the integration of signals generated by multiple TFs to determine the transcriptional output of a given gene. This chapter discusses these themes and some of the experimental techniques used to study the regulation and function of TFs, highlighting specific neuroendocrine-associated examples. We focus on (i) the diversity of function for REST, a TF with roles in neuroendocrine physiology and oncogenesis, (ii) the transcriptional cascades that drive development of the hypothalamic-pituitary axis, and (iii) context-dependent TF function at the gene promoter for the neuropeptide AVP.

1) The key players in transcriptional regulation

1.1 Core transcriptional complexes

Mammalian transcription relies on three multi-subunit core RNA polymerases. RNA pol I and III regulate expression of ribosomal and transfer RNAs respectively. Most pertinent here, RNA pol II regulates expression of messenger RNA (mRNA) coding for proteins, microRNA (miRNA) and long non-coding RNA (lncRNA). The TATA binding protein (TBP) and a host of other general transcription factors are required to correctly position RNA pol II on gene promoters and to support efficient transcriptional initiation. In addition, another multi-protein complex called Mediator is universally required to function as an adapter between these general transcription factors, RNA pol II, and the sequence-specific transcription factors (TFs) that ultimately determine transcriptional output.

1.2 Sequence-specific transcription factors (TFs)

Recent attempts to comprehensively catalog all the human or murine sequence-specific TFs estimate the total number at between 850 and 1900 (Fulton *et al.*, 2009,Vaquerizas *et al.*, 2009). A significant proportion of these TFs are at present completely uncharacterized. Many different TFs have been implicated in regulating the expression of neuropeptides and their cognate receptors, or in driving neuroendocrine development or carcinogenesis; some key examples are listed in Table 1. A global survey of sequence-specific TF mRNA expression shows they comprise approximately 6% of the expressed genes in all tissues, with between 150 and 300 different TFs expressed in any individual tissue. These TFs fall into two general categories, those that are expressed ubiquitously throughout the tissues of the body, and those that are restricted to one or two specific tissues (Vaquerizas *et al.*, 2009). Examples of TFs involved in neuroendocrine processes fall into both of these expression categories (*Extended Table 1*).

All sequence-specific TFs have two major types of domain, which act independently. The first is a DNA-binding domain (DBD) that mediates direct binding of TFs to specific DNA regulatory elements. TFs are classified into more that seventy families on the basis of the specialized DBDs they utilize (Fulton *et al.*, 2009,Luscombe *et al.*, 2000). TFs use these structured domains to probe the topography of the DNA double helix, most commonly the major groove, until they recognize the specific pattern of bases that represents their preferred binding motif. Most TFs employ an α-helix within their DBD for this purpose, and variation in the amino acid (aa) sequence of the DBD generates the differential base specificity of individual TFs. A single DBD element typically recognizes only a very short motif of several base pairs (bp) in length, which would be inadequate to provide suitable specificity within a mammalian genome. However, the scope of these recognition motifs is often extended, either by employing multimerized arrays of binding domains within the TF, or by dimerization between two TFs allowing them to recognize a longer, often inverted, repeat.

Historically, the gene-by-gene empirical determination of DNA sequences bound by a TF was used to define their canonical binding motifs. This

employed techniques such as DNA footprinting, electrophoretic mobility shift assay (EMSA) and reporter gene assays. However, technological advances now enable us to map the binding sites for a given TF across an entire mammalian genome. Methods include (i) computational predictions using motif searches, (ii) protein binding microarrays, and (iii) systematic evolution of ligands by exponential enrichment (SELEX), or (iv) chromatin immunoprecipitation (ChIP), followed by either microarray analysis (ChIP-chip) or next generation sequencing (ChIP-seq).

SELEX is an *in vitro* method used to identify the DNA sequence binding preferences for a TF within a pool of random oligonucleotides, whilst ChIP enables a snapshot of *in vivo* binding by cross-linking TFs at their physiological binding sites within the cellular chromatin environment. High throughput SELEX has now defined binding motifs for over 200 human TFs; although the structural families of TFs as classified by their DBDs do have distinct binding preferences, more precise binding profiles can be used to sub-classify families (Jolma *et al.*, 2013). These approaches often reveal surprisingly wide-scale and diverse binding sites for TFs, and may uncover novel physiological roles through ontology and pathway profiling of the datasets. There are caveats to these studies though. Firstly binding is highly dependent on the physiological context; computation predictions and SELEX cannot account for the cellular environment, whilst ChIP data is specific to the cell line used. Secondly, *in vivo* occupancy of sites does not always equate to transcriptional output. Some estimates suggest that only 25% of TF binding sites identified by ChIP-seq in mammalian cells are linked to transcriptional activity (Spitz & Furlong, 2012). Expression profiling of the TF-responsive transcriptome by microarray or next generation sequencing of RNA (RNA-seq) therefore remain key to understanding the physiological relevance of TF binding.

The major functions of a TF are often conserved across species. However TF binding motifs within DNA evolve rapidly, and the overlap between the human and mouse genomes may be as low as 10% (Vaquerizas *et al.*, 2009). This chapter predominantly discusses TFs with human and rodent orthologs, likely to perform similar functions within neuroendocrine systems. However, their genome-wide binding profiles may vary significantly between species. The DBD targets a TF in a promoter-dependent fashion. This enables precise control of the expression of individual genes, as TFs have a second class of domain that mediates protein-protein interactions to recruit transcriptional co-factors to the gene promoter. Many co-factors bear functional domains that can enhance or repress the activity of the core transcriptional complexes (Figure 1).

1.3 Transcriptional co-factors

Genomic DNA is packaged by nucleosomes, composed of the core histone proteins H2A, H2B, H3 and H4. Less structured “tails” of each histone protrude from the complex, and aa-residues within the tails are targeted for post-translational modification (PTM). These PTMs form a complex histone code that alters the dynamic chromatin environment, rendering it more or less accessible to general and sequence-specific TFs. The code is determined by

many variables including the type, number and location of the PTMs, and the position of the nucleosome within the gene architecture (Li *et al.*, 2007). The writers, readers and erasers of the histone code are transcriptional co-factors. Most sequence-specific TFs can interact with a wide gamut of co-factors, either simultaneously recruiting a large complex with multiple activities towards chromatin, or using alternative co-factors to provide spatial or temporal context to their activity.

Many co-factors possess enzymatic activities that add or remove histone PTMs. For example, addition of acetyl groups by histone acetyl transferases (HATs) opens up the chromatin structure and is associated with transcriptional activation, whilst their removal by histone deacetylases (HDACs) leads to chromatin condensation and transcriptional repression. In contrast, methylation presents a more complex code: increasing methylation of histone H3 on lysine 4 (H3K4) is associated with transcriptional activation, whilst H3K9 or H3K27 methylation is repressive. Families of methyl transferases and demethylases mediate these reversible modifications. Histone readers are recruited to the modified histone residues to act as scaffolds that bring in additional co-factors, ensuring an orchestrated progression of modifications to determine whether a gene is transcribed or repressed.

The ATP-dependent chromatin remodeling SWI/SNF complexes also modulate transcription. As nucleosome positioning influences TF occupancy at enhancers, nucleosome displacement may be required to expose low-affinity TF binding sites. Components of the remodeling complexes, such as BRG1 or BAF, may be recruited as transcriptional co-factors by pioneer TFs, which prime the promoter for binding of other TFs (Spitz & Furlong, 2012).

Intriguingly, some non-coding RNAs act as novel classes of transcriptional co-factor. Mechanistically, lncRNAs may act as signals that mimic TFs, decoys that titrate TFs away from DNA, guides that recruit co-factors in the absence of TFs, or scaffolds that bring together multiple TFs and/or co-factors at chromatin (Wang & Chang, 2011). Small modulatory double-stranded RNAs (smRNAs) of around 20bp in length can also act as TF decoys by mimicking binding motifs, or may modulate interaction between the TF and its co-factors.

2) Classes of neuroendocrine-associated TFs

As summarized in Table 1, TFs that regulate the neuroendocrine phenotype fall into many different classes based on their DBDs. *Full names and further details for all of these TFs can be accessed through Extended Table 1 on the associated website.* Here we briefly overview selected TFs, highlighting their DNA binding preferences and roles in neuroendocrine physiology.

2.1 Basic leucine zipper (bZIP)

The bZIP domain forms a long continuous α-helix consisting of two functional halves. The first is a basic region that makes contact with the DNA, typically

recognizing a short sequence of 4-bp to 5-bp. The second mediates dimerization through formation of a coiled-coil structure. Homodimerization dictates that the active TF recognizes an inverted repeat, but heterodimerization generates alternate factors that recognize distinct asymmetrical binding sites. Key examples of neuroendocrine-associated bZIP TFs are the FOS and JUN family, which heterodimerize to constitute the AP1 transcription factor, and the CREB/ATF family.

CREB1 binds as a homodimer to an 8-bp palindrome known as the cAMP response element (CRE) and is the textbook example of a TF whose activity is controlled by phosphorylation. In response to cAMP signaling, protein kinase A (PKA) is activated, phosphorylating CREB1 on serine-133. CREB1 then translocates into the nucleus and interacts with its co-factor CREBBP to activate target gene transcription. CREB1 is co-activated by a family of TORCs, with TORC1 and TORC2 most highly expressed in the parvocellular and magnocellular neuroendocrine hypothalamus. TORCs are phosphorylated and held in an inactivate state in the cytoplasm by 14-3-3 proteins; when dephosphorylated they move into the nucleus to interact with CREB1, facilitating its interaction with the transcriptional complex. This may be a requirement for CREB-dependent activation, for example corticotrophin releasing hormone (CRH) transcription requires both phosphorylation of CREB1 and nuclear translocation of TORC2 (Aguilera & Liu, 2012). In contrast, CREB3L1 is normally sequestered in the endoplasmic reticulum membrane, from where it is cleaved in response to inducing stresses, allowing translocation into the nucleus to activate transcription. CREB3L1 was recently shown to play a pivotal role in osmotic induction of arginine vasopressin (AVP) expression (section 4).

2.2 Basic helix-loop-helix (bHLH)

The bHLH factors also utilize a basic α-helix to contact DNA, typically binding a 6-bp E-box motif (CANNTG), the canonical form of which is the palindromic sequence CACGTG. The bHLH factors are obligate dimers, and a flexible loop region connects their DNA-binding helix to a second α-helix that enables dimerization. Although homodimerization does occur, heterodimerization is more common and interaction with different dimerization partners provides diversity in sequence recognition and co-factor recruitment.

The transcriptional activator ASCL1 has roles in neural and neuroendocrine progenitor development. ASCL1 is expressed at high levels in human neuroendocrine cancers and forced overexpression of Ascl1 is sufficient to drive development of neuroendocrine lung cancers in mice (Linnoila *et al.*, 2000). In mouse embryonic brain (E12.5) or cultured neural stem cells, genome-wide ChIP-chip identified binding sites for Ascl1 in ~1200 proximal promoters. Enriched amongst these, were genes controlling the neurotransmitter biosynthetic process. Combining these data with expression profiling revealed transcriptional targets that both drive neuronal differentiation and promote cell cycle progression (Castro *et al.*, 2011). Like ASCL1, NEUROD1 and USF2 are also expressed in neuroendocrine cancers. Physiologically NEUROD1 is required for specification of pituitary corticotropes, pathologically it is implicated in a positive feedback loop in

small cell lung cancer (SCLC), as it is upregulated in response to nicotine and increases nicotinic acetylcholine receptor subunit transcription. USF1 and USF2 predominantly heterodimerize, but also form homodimers with distinct binding specificities (Rada-Iglesias *et al.*, 2008). USF2 is overexpressed in SCLC and promotes proliferation (Ocejo-Garcia *et al.*, 2005), whilst USF1/USF2 regulate expression of neuropeptides including AVP, calcitonin gene related peptide (CGRP) and preprotachykinin (PPT-A) (Coulson *et al.*, 1999a,Coulson *et al.*, 2003,Paterson *et al.*, 1995,Viney *et al.*, 2004).

A subfamily of bHLH-PAS factors combines this bHLH domain with PAS (Per/Arnt/Sim) domains that can bind small molecules or other proteins to sense and respond to environmental signals. A heterodimer of two bHLH-PAS factors ARNT2/SIM1 play key roles in hypothalamic development, whilst CLOCK/BMAL1 and HIF1A contribute to regulation of the AVP promoter (section 4).

2.3 Forkhead (FOX)

The forkhead or winged-helix domain is a distinct DBD of around 100-aa, and FOX factors bind to DNA as monomers. The hepatic factor FOXA2 plays roles in developmental systems and is implicated in regulation of neuropeptide gene expression. FOXA2 is a pioneer factor that opens up compacted chromatin to enable binding of other TFs including nuclear receptors (Kaestner, 2010). It also works in a co-operative fashion with USF factors to activate transcription of CGRP (Viney *et al.*, 2004).

2.4 Homeoboxes

There are more than 300 homeobox genes of different sub-classes encoded by the human genome, many of which are associated with developmental processes. They are characterized by a helical DBD, which is essential for function, and are divided into further sub-classes according to their other protein domains. Functions of these TFs in the neuroendocrine hypothalamic-pituitary axis are described in section 4.

2.4.1 POU homeoboxes

Fifteen homeoboxes belong to the POU (Pit1/Oct/Unc86) subclass. They utilize two DBDs, an N-terminal POU-specific domain (~75-aa) that is separated from the C-terminal homeobox domain (~60-aa) by a non-conserved region (5-aa to 20-aa). Each domain uses a helix-turn-helix motif to contact 5-bp to 6-bp of DNA, and both are required for high affinity DNA binding. Many of these factors have roles in neuroendocrine systems, in particular the class I factor POU1F1 (PIT1) that binds the motif TAAAT, and the class III factor POU3F2 (BRN2) (Prince *et al.*, 2011).

2.4.2 PRD homeoboxes

The PRD class is characterized by a serine residue at position 50 that dictates binding specificity and a second conserved PAX DBD. The PRD-like factors have a very similar homeobox, but lack these two key features. A number of PRD (e.g. PAX4, PAX6) and PRD-like factors (e.g. HESX1, OTP, PITX1, PITX2, PROP1) are involved in neuroendocrine development.

2.4.3 NKL homeoboxes

The NKL class genes originate from the NK homeobox cluster in *Drosophila* and often contain an upstream TN motif. HMX2, HMX3 and NKX2-1, which participate in hypothalamic development, serve as examples of this class.

2.4.4 LIM homeoboxes

LIM homeodomain factors contain, in addition to a central homeobox, two N-terminal cysteine-rich LIM domains that mediate protein-protein interactions. Examples include LHX3 and LHX4 that participate in pituitary development.

2.5 T-box (TBX)

The TBX domain is quite large at around 20kDa and is structurally distinct from other DBDs. TFs of this family bind to the DNA consensus sequence TCACACCT. These TFs are mainly involved in developmental processes and TBX19 is required for differentiation of pituitary corticotropes (section 4).

2.6 High mobility group box (HMG-box)

The HMG-box domain contains three α-helices, separated by loops, that make contact with DNA in the minor groove. High affinity HMG-box binding is restricted to unwound DNA conformations. SOX3 acts as a developmental switch, counteracting the activity of proneural factors to suppress neuronal differentiation. It is required for formation of the hypothalamic-pituitary axis (section 4). SOX10 is also associated with neuroendocrine tissues; it is expressed in pulmonary neuroendocrine carcinoids and is implicated in development of gonadotrophin releasing hormone (GnRH) cells in Zebrafish (Whitlock *et al.*, 2005).

2.7 Nuclear hormone receptor (NR)

These TF sensors of steroids and other hormones typically have a C-terminal ligand-binding domain and an N-terminal activation domain, which is ligand-dependent. The central DBD is comprised of two zinc fingers (ZFs) and binds to the hormone response element (HRE). NRs are held in an inactive state in the cytosol and, on ligand sensing, move into the nucleus and bind directly to DNA, either as monomers or as dimers. For example, the glucocorticoid receptor NR3C1 recognizes inverted repeats of a 6-bp DNA motif that are separated by a 3-bp spacer. NR3C1 requires chromatin remodeling by BRG1, a component of the SWI/SNF complex, to access many of its binding sites. In this context, FOXA2 or AP1 may act as pioneer factors to enable chromatin remodeling on which NR3C1 recruitment is dependent (Spitz & Furlong, 2012). The lncRNA GAS5 acts as a decoy for NR3C1 as its stem-loop structure mimics the glucocorticoid response element to which NR3C1 normally binds (Kino *et al.*, 2010). NR3C1 has pervasive roles in neuroendocrinology and may also interact with other transcription factors, altering expression of their responsive genes. Another NR factor, NR5A1 (SF1) is required for development of the adrenal gland, gonads and pituitary gonadotropes. Intriguingly, NR5A1 not only binds its own canonical motif, CAAGGHCA, but can also occupy the RE1 motif used by the ZF repressor REST (Doghman *et al.*, 2013).

2.8. Zinc finger (ZF)

Zinc fingers are comprised of around 30-aa and co-ordinate a single zinc ion at the base of the finger through pairs of conserved cysteine and histidine residues. Each ZF typically recognizes only 3-bp of DNA, and so they are commonly strung together in sequence to produce larger DBDs. Over 600 human TFs use ZFs to bind DNA. Many examples associated with neuroendocrine regulation primarily act as transcriptional repressors. These either silence neuroendocrine gene expression in non-neuroendocrine tissues, or promote differentiation by switching off expression of genes that suppress neuroendocrine gene expression. Consideration of the preferred DNA binding motifs for some specific ZF factors illustrates that this prevalent DBD can provide diverse recognition profiles for individual TFs within the human genome (Figure 2).

INSM1 is a ZF repressor whose expression is tightly restricted to endocrine tissues. It has a C-terminal DBD comprised of five ZFs, which recognize a 12-bp consensus motif (Figure 2). INSM1 is transiently expressed during neuroendocrine differentiation and regulates development of the endocrine pancreas, as well as the noradrenergic sympathetic neurons and chromaffin cells of the sympathoadrenal gland. INSM1 is also highly overexpressed in most neuroendocrine cancers (Lan & Breslin, 2009). IKZF1 was originally described as a lymphocyte differentiation factor, although it also influences hypothalamic-pituitary cell development, differentiation, proliferation and transformation (section 4). IKZF1 has a C-terminal interaction domain involved in dimerization and an N-terminal DBD comprised of five ZFs, although its preferential DNA recognition motif is not well established. Interestingly, IKZF1 exists as several alternatively spliced isoforms, most of which lack sufficient ZFs to bind DNA efficiently, and act in a dominant negative fashion. IKZF1 isoforms are expressed in pituitary adenomas, and act as transcriptional activators or repressors for a variety of hormones, such as pro-opiomelanocortin (POMC), growth hormone, (GH), prolactin (PRL) and GH-releasing hormone (GHRH) (Ezzat & Asa, 2008).

SCRT1 is a transcriptional repressor that utilizes five ZFs to bind DNA at E-box motifs, competing with bHLH factors. It is a neural-specific repressor, expressed in newly differentiated post-mitotic neurons, and may mediate a switch to migratory neurons (Itoh *et al.*, 2013). SCRT1 is expressed in neuroendocrine cancers, where it antagonizes the pro-neural bHLH factors ASCL1 and E12 (Nakakura *et al.*, 2001). In contrast SP1 is widely expressed with numerous physiological roles. SP1 has three ZFs that bind GC-rich DNA motifs (Figure 2), and it may act as either a transcriptional repressor or activator. SP1 is associated with transcriptional activation of POMC and GnRH. GATA2, involved in specification of pituitary gonadotropes and thyrotopes, is also quite widely expressed. It possesses a different class of GATA-type ZF, in which four cysteine residues coordinate the zinc ion. These highly conserved DBDs bind to the motif (A/T)GATA(A/G).

An example of a TF that prevents neuroendocrine expression in inappropriate tissues is REST, also known as NRSF. The central DBD of REST consists of eight ZFs, which bind a 21-bp consensus RE1 motif (Figure 2). However, as

discussed below, intensive study of genome-wide REST occupancy finds this motif to be highly divergent and surprisingly prevalent. REST is widely expressed outside the nervous system and was first described as a silencer of neuronal genes in non-neuronal cells (Chong *et al.*, 1995,Schoenherr & Anderson, 1995). However, REST is now known to dynamically regulate a broad spectrum of target genes and is implicated in many facets of the neuroendocrine phenotype (section 3).

3) REST: a zinc finger TF with complex regulation and diverse function

REST controls transcription of vast repertoire of target genes that play key roles in development and normal physiology. REST dysregulation is associated with diseases as diverse as Down's syndrome, epilepsy, neurodegeneration and cancer, where it acts in a context-dependent fashion as either an oncoprotein or a tumor suppressor (Coulson, 2005,Negrini *et al.*, 2013). Importantly, the loss of REST in neuroendocrine lung cancers licences inappropriate expression of neuropeptides, neurosecretory pathway components and neurotransmitter receptors, which can convey growth advantages (Coulson *et al.*, 1999b,Coulson *et al.*, 2000,Gurrola-Diaz *et al.*, 2003,Moss *et al.*, 2009). REST is a bipartite repressor, which recruits a variety of co-factors through N-terminal (RD1) and C-terminal (RD2) repression domains (Figure 3). It is part of the pluripotency network in embryonic stem cells and decreases as progenitors differentiate along a neuronal program, permitting expression of neural-specific transcripts (Ballas *et al.*, 2005). However, REST also controls expression of many other protein-coding mRNAs, as well as regulatory non-coding RNAs, which may act in feedback loops. Perhaps unsurprisingly, its own expression and function is tightly regulated. Here we use REST as a paradigm for the complexity of TF functionality (Figure 4).

3.1 Transcription and alternative splicing of REST

REST function is regulated in many ways, including through altering its transcription, or by alternative splicing that generates isoforms lacking key domains (Figure 3). During neurogenesis, the reduction in REST is partly attributed to abrogation of REST transcription, and this may also be downregulated in SCLC by promoter methylation (Kreisler *et al.*, 2010). However, alternative splicing in neurons, neuroblastoma and SCLC also alters REST function (Coulson *et al.*, 2000,Palm *et al.*, 1998,Palm *et al.*, 1999). A common splice variant retains an internal neural-specific exon and encodes a truncated isoform, known as REST4 or sNRSF, lacking the C-terminal repression domain. Intriguingly, the splicing regulator SRRM4 (nSR100), expressed in both neurons and SCLC, promotes inclusion of this exon and is itself a REST-target gene (Raj *et al.*, 2011,Shimojo *et al.*, 2013). Other splice variants skip a domain required for nuclear translocation (Shimojo *et al.*, 2001), or truncate REST by using an alternative 3' exon (Chen & Miller, 2013).

Although REST4 retains only five of the eights ZFs in the DBD, reducing its binding affinity, it may compete with REST at a subset of RE1 motifs. The

prevalence and consequences of REST isoforms remain under debate. However, the absence of RD2 in REST4 may mitigate repression of target genes. For example, REST4 induction is seen on differentiation of human embryonic stem cells into neural progenitor cells where neuronal gene expression is activated (Ovando-Roche *et al.*, 2014) and in epilepsy models REST4 induction corresponds with that of the neuropeptide PPT-A (Spencer *et al.*, 2006). Further physiological evidence comes from a rodent study into the effect of early life stress on subsequent chronic stress. In this model, as the hypothalamic-pituitary-adrenal axis response increases, both REST4 expression and the transcription of REST target genes are upregulated in the prefrontal cortex (Uchida *et al.*, 2010).

3.2 Post-translational modification, stability and cellular localization

In common with many TFs, the functionality, localization and stability of REST are controlled by reversible PTMs and protein interactions. Mature REST is glycosylated (Lee *et al.*, 2000,Pance *et al.*, 2006), which although still poorly characterized, is associated with nuclear localization. The targeting of REST to the nucleus has also been associated with the fifth ZF that is spliced out in some variants (Shimojo *et al.*, 2001), or by the interacting proteins RILP (PRICKLE1), p150-glued (DCTN1) and huntigtin (HTT) (Shimojo & Hersh, 2003,Shimojo, 2011). In addition to relocalization, REST activity is also controlled by acute ubiquitin-mediated proteasomal degradation.

REST becomes acutely phosphorylated during neural differentiation, cell division and adenoviral infection. Using mass spectrometry, this has been mapped to two independent phosphodegrons close to the C-terminal repression domain. Several candidate kinases have been suggested. The Down's syndrome-associated kinase DYRK1A, a transcriptional target of REST, interacts with the REST-SWI/SNF complex, potentially establishing a negative feedback loop (Lu *et al.*, 2011), whilst casein kinase (CK1) phosphorylates REST in adult neurons (Kaneko *et al.*, 2014). Activation of REST phosphodegrons triggers acute polyubiquitylation of REST by the E3 ligase $SCF^{\beta TrCP}$ (BTRC) leading to its degradation (Guan & Ricciardi, 2012,Guardavaccaro *et al.*, 2008,Westbrook *et al.*, 2008). In the case of neural differentiation, REST degradation is antagonized by the deubiquitylase USP7 (Huang *et al.*, 2011). Interestingly, different REST isoforms lack residues required for either phosphorylation or interaction with USP7 (Figure 3). REST protein abundance changes during the cell cycle, notably at the G2/M and M/G1 transitions; REST degrades as cells enter mitosis but rapidly recovers at mitotic exit. We recently identified the deubiquitylase USP15 as a regulator of REST stability by siRNA screening. Using mitotic and translational inhibitors we demonstrated that USP15 specifically promotes new REST synthesis (Faronato *et al.*, 2013). Intriguingly, USP15 expression is relatively low in post-mitotic neurons, but is amplified in glioblastoma (Eichhorn *et al.*, 2012) where REST has oncogenic function (Kamal *et al.*, 2012).

Another player in the regulation of REST activity is the telomere repeat protein TRF2. In pluripotent cells, TRF2-REST complexes are sequestered in aggregated nuclear PML bodies and protected from proteasomal degradation.

However, during development, there is a switch in TRF2 isoforms, which now sequester REST in the cytoplasm, leading to derepression of target gene expression and promote acquisition of the neuronal phenotype. Intriguingly, TRF2 also binds to the C-terminal of the REST4 isoform protecting its stability in neural progenitor cells (Ovando-Roche *et al.*, 2014,Zhang *et al.*, 2008,Zhang *et al.*, 2011).

3.3. REST Transcriptional co-factors

REST recruits a diverse cohort of transcriptional co-factors (Figure 3). For an extensive discussion of this topic and full referencing we refer the reader to two comprehensive reviews (Bithell, 2011,Ooi & Wood, 2007). Here we focus on the emerging understanding of their co-operative functions and the significance of alternative REST co-factor complexes.

3.3.1 Protein co-factors

Yeast two-hybrid screening has identified two major REST co-repressor complexes: SIN3A/B that binds RD1 serving as a docking site for HDAC1/2 (Grimes *et al.*, 2000,Huang *et al.*, 1999), and RCOR1 (coREST) that binds RD2 (Andres *et al.*, 1999). RCOR1 was subsequently shown to recruit many histone modifiers that contribute to the repressive chromatin environment. These include HDAC1/2 and BHC80, the demethylases LSD1 (H3K4me/me2) and KDM5C (JARID1C or SMCX, H3K4me2/me3), the methyl transferases EHMT2 (G9a, H3K9me2) and EZH2 a component of the polycomb repressive complex PRC2 (H3K9 and H3K27). Intriguingly, whilst both RD1 and RD2 must be retained for full repression of some target genes, a single repression domain is sufficient to repress others; this is important when considering the activity of isoforms like REST4. Both full-length REST and RCOR1 can also interact with components of the ATP-dependent chromatin-remodeling complex, including BRG1 (SMARCA4), BAF53 (ACTL6A) and BAF170 (SMARCC2), and with the methyl binding protein MECP2. In addition, REST can block the basal transcription machinery: it binds to TBP inhibiting formation of the pre-initiation complex and SCP1, inhibiting RNA pol II activity.

It is suggested that step-wise recruitment of these co-factors coordinates progressive chromatin changes that ultimately switch off expression of target genes. The nucleosome remodeling activity of BRG1 may be an early requirement, to provide better access and stabilize REST binding at RE1 sites. Profiling of nucleosome positioning and of 38 histone modifications by ChIP-Seq analysis revealed the complexity of the chromatin landscape remodeled by REST (Zheng *et al.*, 2009). This study provides good evidence for co-ordination of histone modifications, as REST binding is often correlated with decreased acetylation (H3K4ac, H4K8ac) and active methylation marks (H3K4me3), but increased repressive methylation (H3K27me3, H3K9me2). However, not all co-factors are recruited to each REST locus concomitantly, and this may vary according to the cellular context (Greenway *et al.*, 2007,Hohl & Thiel, 2005). Thus target genes may acquire different chromatin modifications as a consequence of REST binding. The selective engagement of co-factors may be linked to the strength and dynamics of binding and repression, such that alternative co-factor complexes may distinguish between transient repression and long term silencing mechanisms. In this

context, MECP2 recognizes repressive methylation marks within CpG islands and can retain repression at promoters once REST is no longer bound (Ballas *et al.*, 2005).

3.3.2 Non-coding RNA co-factors

To date, two ncRNAs have been shown to modulate transcriptional repression by REST through contrasting mechanisms (figure 4). HOTAIR, a lncRNA transcribed from within the HOXC cluster, acts as both a guide and a scaffold, to repress transcription of the HOXD cluster. HOTAIR recruits PRC2/EZH2 through binding to its 5' sequence, and the LSD1/RCOR1/REST complex at its 3' sequence; this molecular bridge co-ordinates H3K27 methylation by EZH2 with H3K4 demethylation by LSD1. Interestingly, this HOTAIR-REST complex now uses the right-hand RE1 half-site to bind DNA, potentially altering its profile of target genes (Tsai *et al.*, 2010). In contrast, a double-stranded smRNA found in neurons mimics the RE1 binding site for REST and results in transcriptional activation of REST target genes, specifying the fate of adult neural stem cells. However, this smRNA does not act as a decoy, as ChIP analysis shows REST still binds to target gene promoters, but without recruitment of its usual co-repressors. The smRNA was therefore suggested to switch the function of chromatin-associated REST from that of a repressor to a transcriptional activator (Kuwabara *et al.*, 2004).

3.4. Diversity of transcriptional targets

3.4.1 Genome-wide RE1 identification

REST has proved of particular interest for genome-wide profiling, due to the long recognition motif for its DBD (Figure 2). Numerous studies attempted to predict RE1 binding sites (reviewed in (Bithell, 2011,Ooi & Wood, 2007)). However, early empirical global studies revealed many more binding sites than expected. One used serial analysis of chromatin occupancy (SACO) in human lymphocytes; the other, in mouse kidney cells, was the first published ChIP-seq study (Johnson *et al.*, 2007,Otto *et al.*, 2007). The increase in binding sites was partly due to the discovery that the RE1 motif functions as two half sites separated by a spacer, which varies in length from 2bp, found in the most common canonical sequence, up to at least 8bp (Figure 4). Intriguingly, RE1 motifs were later divided into subgroups that are human, primate, or mammal-specific, and a small group that are deeply conserved across reptiles, amphibians and fish (Johnson *et al.*, 2009). On comparison with the mouse genome, human RE1 motifs fell into three equal groups that either aligned to mouse RE1, or aligned with the mouse genome despite the lack of a murine RE1, or failed align with mouse genome at all. The most recent compilation across global occupancy studies, suggests up to 21,000 REST binding sites within the human genome (Rockowitz *et al.*, 2014).

Broadly speaking, REST binding at both canonical and expanded RE1 motifs correlates with loss of transcription and occurrence of the expected histone marks (Zheng *et al.*, 2009). However, some studies suggest that only half of REST occupancy sites recruit co-factors (Yu *et al.*, 2011). The sequence context around an RE1 influences co-factor recruitment, and specific cofactors mark higher (SIN3A) or lower (EZH2) expressed targets (Rockowitz *et al.*, 2014). It is clear that REST occupancy is dynamic and depends on the

cellular context. For example, tumor suppressors are identified as targets in cancer cells, but a very different profile of REST targets is seen in neurons compared to non-neuronal cells (Rockowitz *et al.*, 2014). Intriguingly, whilst a number of ChIP-validated occupancy sites are not RE1 (Johnson *et al.*, 2008), other TFs may also compete for binding at RE1 motifs. ChIP-seq for SF1 in adrenocortical cells shows enriched occupancy at RE1 in addition to the SF1 consensus site. Indeed, SF1 could relieve REST repression of key steroidigenic genes (Doghman *et al.*, 2013). From a physiological perspective, genome-wide occupancy and transcription analyses concur that REST controls diverse processes, regulating expression of neuropeptides, neurotransmitter receptors, synaptic signaling and neuroendocrine secretion, as well as other TFs that drive neuronal and endocrine differentiation.

3.4.2 Transcriptional targets: mRNAs and non-coding RNAs

REST, via its myriad binding sites, regulates both mRNA and ncRNA expression. REST targets of both classes operate in feedback loops that influence protein expression of target genes, and directly impact on REST function. The contribution of such mechanisms to REST-dependent expression networks is highlighted in Figure 4.

Our interest in REST arose from the discovery that it was a negative regulator of neuropeptides including PPT-A (Quinn *et al.*, 2002) and AVP (section 4). Other neuropeptides and hypophysiotropic hormones are also REST target genes, including CRH (Korosi *et al.*, 2010), establishing REST as a neuroendocrine-associated TF. Indeed ontology analysis from the first global ChIP study identified a role for REST in coordinating neuroendocrine pancreatic development (Johnson *et al.*, 2007). Recently, IL6 was found to induce neuroendocrine differentiation of prostate cancer cells through downregulating USP7 and accelerating REST turnover (Zhu *et al.*, 2014). Targeted transcript analysis and DNA microarray studies of the REST-dependent transcriptome, conducted in REST-deficient PC12 cells, on dominant negative REST expression in neuronal cells, or following siRNA depletion of REST in lung cancer cells, have all highlighted a role for REST in regulating the neurosecretory phenotype (D'Alessandro *et al.*, 2008,D'Alessandro *et al.*, 2009,Hohl & Thiel, 2005,Moss *et al.*, 2009,Pance *et al.*, 2006). Target genes in include many synaptic and dense core vesicle proteins, as well as the chromogranin and prohormone convertase families.

Non-coding RNA is diverse in form and function (*Chapter 4*) and lncRNA and miRNA targets of REST were identified through genome-wide occupancy and microarray studies (Conaco *et al.*, 2006,Gao *et al.*, 2012,Ng *et al.*, 2012,Rockowitz *et al.*, 2014). Most recent data suggests REST occupancy at 14% of currently annotated human miRNAs, with 4.2% of these differential expressed in neurons (Rockowitz *et al.*, 2014). Currently, only a handful of these have been extensively investigated, most notably miR-9 and miR-124. These REST-regulated miRNAs often exert feedback on REST function by targeting REST expression, or its cofactors including SCP1, RCOR1, MECP2 and EZH2, as well as switching neural progenitor BAF53a for neural BAF53b in the chromatin remodeling complex (Packer *et al.*, 2008,Rockowitz *et al.*, 2014,Visvanathan *et al.*, 2007,Wu & Xie, 2006,Yoo *et al.*, 2009). Intriguingly,

several mRNAs that are normally repressed by REST also feedback to regulate REST function, including the splicing factor SRRM4 (Raj et al, 2011) and the kinase DYRK1A (Lu et al, 2011). Developmentally, miRNAs expressed as a consequence of REST downregulation, contribute to establishing neuronal phenotype. For example, in combination with the TFs POU3F2 and MYTL1, miR-124 expression is sufficient to induce conversion of fibroblasts into neurons (Ambasudhan *et al.*, 2011). Cross-regulation of these miRNAs also integrates REST into networks with other neuronal and neuroendocrine TFs such as POU3F2, NEUROD1 and CREB1 (Rockowitz *et al.*, 2014,Wu & Xie, 2006). The context-specific studies published to date provide a glimpse into the extensive feedback between REST and ncRNAs that is proposed to govern maintenance and renewal of neuronal stem cells, differentiation and establishment of neural identity (Qureshi & Mehler, 2012).

4) Cooperation of TFs in neuroendocrine phenotype and function

4.1 Transcriptional networks in neuroendocrine development

Neuronal differentiation is a highly coordinated process during which cells commit to a neuronal fate, acquire positional identities, exit the cell cycle, migrate and terminally differentiate. Key to these processes are cascades of TFs that establish gene expression programs to develop, define and maintain the correct phenotypes. Here we overview the TFs implicated in the development and physiological function of specific cells within the neuroendocrine hypothalamus and the anterior pituitary gland.

4.1.1 Magnocellular and parvocellular neurons of the hypothalamus

The hypothalamus sits below the thalamus and above the pituitary gland, to which it is connected; together they play a major role in homeostasis. Two classes of hypothalamic neurons form functional nuclei. Magnocellular neurons originate in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus and extend their axons into the posterior pituitary. In response to physiological stimuli, they release the neuropeptides oxytocin (OT) and AVP directly into the circulation. In contrast, parvocellular neurons project from hypothalamic nuclei to the median eminence, where they secrete hypophysiotropic hormones. From here, the hypophysial portal system runs down the pituitary stalk into the anterior lobe, where the hormones act on specialized pituitary cells. The parvocellular neurons are classified according to the hormones they produce: CRH and thyroid releasing hormone (TRH) neurons are found in the PVN; somatostatin (SS) neurons in the anterior periventricular (aPeV) nucleus; SS, GHRH and dopamine (DA) neurons in the arcuate (ARC) nucleus; GnRH neurons in the preoptic area (POA) and gonadotropin-inhibiting hormone (GnIH) neurons in the dorsal-medial nucleus (DMN). A number of TFs expressed in the developing hypothalamus were mapped to progressive definition of these neuroendocrine lineages using human disease mutations and rodent models (Figure 5).

Otp is expressed from E10 in the mouse diencephalon, and by E17 is restricted to the regions from which the hypothalamic neuroendocrine nuclei originate (Simeone *et al.*, 1994). Otp is required at multiple stages of

development, from the initial proliferation and migration of progenitor cells, through neuroendocrine differentiation, and during hormone expression from established nuclei. These pervasive and essential roles of Otp are apparent in knockout mice, which fail to form both the magnocellular and parvocellular neurons of the aPeV, ARC, PVN or SON, and lack hypothalamic expression of the neuropeptides CRH, TRH, AVP, OT, SS and DA (reviewed in (Del Giacco *et al.*, 2008). Sim1/Arnt2 act in parallel with Otp and, although not required in progenitor cells, Sim1/Arnt2 mutant mice have a reduced number of hypothalamic cells. These mice fail to establish the SON, lack parvocellular and magnocellular neurons of the PVN, and SS neurons of the aPeV, and ultimately lose production of all these neuroendocrine hormones. Downstream of both Otp and Sim1/Arnt2 is Pou3f2 (also known as Brn2), which is normally expressed in the SON and much of the PVN. Pou3f2 knockout mice do not express CRH, OT or AVP, as they fail to establish the requisite neurons of the SON and PVN, although they do retain expression of TRH and SS (reviewed in (Prince *et al.*, 2011,Szarek *et al.*, 2010)).

Sox3 may be required for proper development of most parvocellular neurons. Sox3 null mice, and human patients with SOX3-linked hypopituitarism disorder, have multiple pituitary hormone deficiencies (Szarek *et al.*, 2010). Although this may also be linked to additional roles for Sox3 in the anterior pituitary itself, where it is required for development but not normal function. Other TFs implicated in development of specific parvocellular nuclei include Ascl1, Ikzf1, Nkx2.1, Hmx2/Hmx3 and Nr5a1. Proneural Ascl1 (also know as Mash1) is broadly required for neurogenesis throughout the central nervous system, and Ascl1 null mice fail to develop the ARC and ventromedial nucleus (VMN) nuclei. Ascl1 is linked to neuronal sub-type specification and, in the context of the hypothalamus, is required for establishment of GHRH expressing neurons. Ikzf1 is also expressed in the developing GHRH neurons, and Ikzf1 knockout mice display severe neuroendocrine phenotypes including dwarfism (Ezzat & Asa, 2008). Nkx2.1 (also known as Ttf1 or T/ebp) was originally described as a thyroid-specific TF, but is also expressed in developing lung and the presumptive hypothalamus. Nkx2.1 mutant mice die at birth, exhibiting lung, thyroid and ventral hypothalamus defects, specifically in the ARC and VMN. Two closely related TFs, Hmx2 and Hmx3, may have redundant functions in hypothalamic development. Mice that are null for both Hmx2 and Hmx3 have a severe deficiency of GHRH neurons in the ARC, but not the VMN, and exhibit dwarfism. Lastly, Nr5a1 (also known as SF1) is required for development of the adrenals, gonads and pituitary gonatotropes. Within the hypothalamus, Nr5a1 expression is restricted to the VMN, and is broadly required from the initial growth and migration of VMN precursors, to their terminal differentiation (Szarek *et al.*, 2010).

The downstream transcriptional pathways for many developmentally important TFs remain incompletely characterized. However, these and other TFs directly regulate transcription of neuropeptides or hypophysiotropic hormones. For example, the CRH promoter is directly repressed by REST (Korosi *et al.*, 2010) and activated by POU3F2 and CREB1 (Aguilera & Liu, 2012), IKZF1 induces GHRH transcription (Ezzat & Asa, 2008) and NKX2-1 is a transcriptional regulator of GnRH.

4.1.2 TFs that specify the anterior pituitary

In contrast to the posterior pituitary, the anterior pituitary is a true gland. Cells of the anterior pituitary fall into five distinct subtypes: gonadotropes that produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH); thyrotropes that produce thyroid-stimulating hormone (TSH); lactotropes that produce PRL; somatotropes that produce GH; and corticotropes that synthesize POMC which is processed into adrenocorticotropic hormone (ACTH). These pituitary hormones are released under control of the hypothalamic parvocellular neurons, as receptors on the pituitary cell surface recognize the appropriate hypophysiotropic hormone, which increases or decreases their hormone secretion into the general circulation. Mutations in several TFs result in impaired pituitary function, and transgenic models have clarified the cascades of TFs that specify development of anterior pituitary cells. Development of the human pituitary follows a similar, although not identical program, and disease-associated mutations in human patients suggest the TF ortholgs play similar roles. A simplified overview highlighting some key TFs in this developmental network is shown in Figure 6.

The anterior pituitary is derived from Rathke's pouch, an invagination of the oral ectoderm, under the control of a series of signaling pathways (reviewed in (de Moraes *et al.*, 2012)). Several homeobox TFs are required early in this process. Pitx1 (also called Tpit) and Pitx2 are expressed in Rathke's pouch and persist in the gonadotropes and thyrotropes of the adult pituitary. Lhx3, and the related Lhx4, are key regulators of anterior pituitary cell commitment and differentiation, being required for early development of Rathke's pouch. Experiments in knockout mice show that pituitary expression of Lhx3 is dependent on both Pitx1 and Pitx2. Lhx3 also persists in the adult pituitary, where it directly activates transcription of various pituitary hormones and other regulatory TFs.

HesX1 is present before Rathke's pouch forms and its downregulation is required for anterior pituitary cell differentiation. HesX1 negatively regulates pituitary-specific Prop1, first expressed at E10.5. Prop1 mutation is responsible for the hypoplastic pituitary phenotype of the Ames dwarf mouse, which lacks expression of GH, TSH, PRL, LH and FSH. Prop1 regulates downstream expression of another pituitary-specific TF, Pou1f1 (Pit1), which is expressed in mice from E13.5 through to adulthood. Pou1f1 specifies thyrotropes, lactotropes and somatotropes, all of which are lacking in dwarf mice with Pouf1 mutation. It regulates transcription of many genes within these lineages, including GH, PRL and TSHβ; human patients with POU1F1 mutations are deficient in these same neurohormones (Prince *et al.*, 2011). Additional TFs including Nr5a1, Gata2, Izkf1, Tbx19 and NeuroD1 are required later in differentiation to specify hormone-secretory pituitary cell types (Figure 6). For example, Ikzf1 expression is important for anterior pituitary cell growth, differentiation and survival. Ikzf1 directly regulates POMC expression in co-operation with PitX1 by recruiting the co-activator SRC/P160, and increases PRL but decreases GH expression (Ezzat & Asa, 2008). Whilst POMC processing relies on a number of convertases, including PCSK1, which is transcriptionally repressed by REST (Moss *et al.*, 2009).

4.2 Context-dependent regulation of the AVP promoter

To conclude this chapter, we will briefly consider the context-dependent expression of the neuropeptide AVP. In a normal physiological context, AVP is transcribed in and released from magnocellular neurons of the SON and PVN in response to changes in osmolality, and acts on AVP receptors in the kidneys and blood vessels to maintain homeostasis. AVP is also transcribed in the suprachiasmatic nucleus (SCN) in response to circadian cues. However the AVP gene was first cloned and sequenced from a SCLC cell line (Sausville *et al.*, 1985) and is commonly overexpressed in these neuroendocrine tumors, where it and can lead to the syndrome of inappropriate secretion of anti-diuretic hormone (SIADH) and dilutional hyponatraemia (Johnson *et al.*, 1997).

A decade ago, we reviewed the binding motifs and TFs that regulated pathological expression of the AVP gene promoter in SCLC, highlighting roles for loss of repression by REST through an RE1 motif at the transcriptional start site, and activation by USF1/USF2 through proximal E-box motifs (Coulson, 2002). Interestingly, whilst USF1/USF2 bind the major E-box of the AVP promoter in SCLC, the bHLH-PAS heterodimer CLOCK/BMAL1 (Jin *et al.*, 1999) utilizes this same E-box during circadian regulation of AVP transcription in the SCN. Another bHLH-PAS factor, HIF1A, mediates crosstalk between hypoxic and circadian signaling by promoting BMAL1 recruitment (Ghorbel *et al.*, 2003). Although the physiological transcription of AVP in the magnocellular neurons is induced by hyperosmotic stress and cAMP signaling, until recently it remained unclear which TFs mediated this response. New studies found no direct role for CREB1, but instead show a key role for CREB3L1. Both transcriptional induction and cellular relocalization of CREB3L1 are seen in response to osmotic challenge, and CREB3L1 can bind and activate the AVP promoter (Greenwood *et al.*, 2014). The TFs that have been physically mapped to the AVP promoter are summarized in Figure 7.

5) Perspectives

The complex networks that regulate transcription of physiological processes are slowly being uncovered. Systems biology approaches are required to understand how these transcriptional networks are integrated, but we do not yet know the full complement of TFs encoded by the human or murine genomes. Study of even a single TF reveals unexpected complexity, with multiple levels of regulation that contribute to contextual differences in their transcriptional activity. Considerable advances in the techniques available to study TFs are enabling their roles to be established in different tissues, through development, and in response to specific stimuli. Genome-wide maps of TF occupancy are helping to build networks, but this is hampered by the inter-species evolution of binding sites, and the incomplete correlation of binding with TF activity. Given these limitations, mapping transcriptional regulation of the neuroendocrine phenotype remains a work in progress.

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7) Glossary

Histone: A small highly conserved basic protein, found in the chromatin of all eukaryotic cells.

Nucleosome: The basic unit of chromatin that contains 147-bp of DNA wrapped around a histone octamer.

Chromatin: The genomic DNA, histone proteins and other closely-associated non-histone proteins.

ATP-dependent chromatin remodeler: Large multi-subunit molecular machine that uses ATP energy to reorganize nucleosome structures, often by sliding the nucleosome to a new position on the DNA.

Gene promoter: the region of a gene, usually immediately 5' to the transcriptional start site, which recruits multiple transcription factors.

Enhancer element: a region of the gene that binds activating transcription factors.

Silencer element: a region of the gene that binds repressing transcription factors.

Pioneer factors: proteins that can penetrate condensed chromatin to pioneer recruitment of secondary co-factors that remodel the chromatin to allow other TFs access.

Post-translational modification: the additional of a small molecule or peptide onto a protein after its translation is complete; modifications are usually reversible and regulatory.

Gene Ontology: A universal classification system of gene functions and other attributes that uses a controlled vocabulary.

Ortholog: Loci in two species that are derived from a common ancestral locus by a speciation event.

Transcriptome: the full complement of transcripts produced in the cell or tissue under investigation.

Microarray: The use of high-throughput hybridization technology for transcriptomic profiling.

RNA-seq: The use of high-throughput sequencing techniques for transcriptomic profiling.

DNA Footprinting: A technique to detect protein–DNA interactions using an enzyme to cut DNA, followed by analysis of the resulting cleavage pattern to identify the footprint that the protein protects.

EMSA: A technique that uses native gel electrophoresis to determine whether, and how specifically, a protein of interest can bind a given DNA sequence.

SELEX: A combinatorial technique for producing DNAs that bind specifically and with high affinity to a DNA-binding protein of interest.

ChIP-chip: Combines chromatin immunoprecipitation (ChIP) with microarray (chip); a high-throughput method for genome-wide identification of DNA regions that are bound *in vivo* by a target protein of interest.

ChIP-seq: Similar to ChIP–chip, but interacting DNA motifs are read out by high-throughput parallel sequencing.

8) Figure Legends and Tables

Figure 1. Key concepts: generalized pathway by which sequence-specific transcription factors direct physiological processes.
The expression, cellular localization and activity of transcription factors (TFs) are tightly controlled. When in an active state, TFs are targeted to bind certain gene promoters through recognition of specific DNA motifs. TFs recruit a variety of co-factor complexes, which alter the chromatin environment around the target gene to activate or repress basal transcription. TFs direct expression of both messenger RNAs (mRNA) that encode proteins and non-coding RNAs (ncRNAs) that modulate protein expression through different mechanisms. Integration of signals at a promoter determines whether the target gene is expressed, and this feeds into larger expression networks.

Figure 2. Examples of binding motifs for neuroendocrine-associated zinc finger TFs.
The position weight matrices derived by ChIP-seq (JASPAR, http://jaspar.genereg.net) are shown for three TFs that use DNA binding domains with different configurations of zinc fingers to determine their binding specificities: REST (8 ZF), INSM1 (5 ZF) and SP1 (3 ZF).

Figure 3. REST isoforms and co-factors.
The major REST isoforms has two repression domains RD1 and RD2 that recruit differential transcriptional co-factor complexes. Alternative splicing potentially generates multiple REST isoforms lacking key domains, which may antagonize REST function. Examples shown are numbered according to Uniprot (http://www.uniprot.org/uniprot/Q13127). Truncated isoforms are generated by inclusion of a neural-specific exon between exons 3 and 4 (isoforms 2 and 3) or the use of an alternative 3' exon 5 (Chen & Miller, 2013), these lack several ZFs of the DBD, RD2 and the phosphodegron. ZF5 of the DBD domain, which recruits USP7 and mediates nuclear localization, is deleted in isoforms 2 and 4.

Figure 4. REST as a paradigm for diversity and feedback in transcription factor regulation and function.
REST binds to a diverse array of RE1 motifs and recruits co-repressors (green) to switch off transcription. In the absence of REST, transcription is enabled that promotes the neuronal/neuroendocrine phenotype. Target mRNAs include regulatory proteins (orange) and miRNAs (blue) that establish feedback loops with REST. Other TFs (purple) may compete for RE1, and ncRNAs modulate REST interactions with the RE1 and protein co-factors. Grey lines show protein interactions and blue lines show ncRNA interactions.

Figure 5. TFs required for development of the neuroendocrine hypothalamus.
Examples of TFs that promote early commitment and later differentiation of the hypothalamic magnocellular and parvocellular neurons.

Figure 6. TF cascades in anterior pituitary development.
Examples of TFs that promote early commitment and later differentiation of anterior pituitary cells.

Figure 7. Context-dependent TF regulation of the AVP promoter.
Examples of TFs that activate or repress transcription through the AVP proximal promoter in response to osmotic, circadian or pathological cues.

Table 1. Examples of sequence-specific transcription factors associated with regulation of neuroendocrine phenotype.
HGNC human names are listed, with common names in brackets.
See associated website for extended table with further details and external links.

TF name: HUMAN / Mouse	Alternative names	DBD Type	Tissue expression profiles	External Databases
ATF1 Atf1	Activating transcription factor 1, TREB36	bZIP	Smooth muscle, whole blood, IJV (1). EMBL Atlas	Genecards AnimalTFBD
CREB1 Creb1	cAMP responsive element binding protein 1, CREB	bZIP	Appendix, testis, whole blood, IJV (1). EMBL Atlas	Genecards AnimalTFDB TFe
CREB3L1 Creb3l1	cAMP responsive element binding protein 3 -like protein, OASIS	bZIP	General (1). EMBL Atlas	Genecards AnimalTFDB TFe
FOS Fos	FBJ murine osteosarcoma viral oncogene homolog, AP-1, C-FOS	bZIP	Bone marrow, lung, thyroid, trachea (1). Stress-inducible. EMBL Atlas	Genecards AnimalTFDB TFe
JUN Jun	Jun proto-oncogene, AP1, C-JUN	bZIP	Lung, pancreas, prostate, thyroid, uterus (1). EMBL Atlas	Genecards AnimalTFDB TFe
NR3C1 Nr3c1	Nuclear Receptor Subfamily 3, Group C, Member 1, Glucocorticoid receptor, GR	Nuclear receptor	Smooth muscle, whole blood, JJV (1). EMBL Atlas	Genecards AnimalTFDB TFe
NR5A1 Nr5a1	Steridogenic factor 1, SF1, FT2F1	Nuclear receptor	Sex differentiation, pituitary gonadotrope and hypothalamic VMN development. EMBL Atlas	Genecards AnimalTFDB TFe
LHX3 Lhx3	LIM Homeobox 3, CPHD3, LIM3	LIM homeobox	Pituitary (1). EMBL Atlas	Genecards AnimalTFDB TFe
LHX4 Lhx4	LIM Homeobox 4, CPHD4	LIM homeobox	EMBL Atlas	Genecards AnimalTFDB TFe

HMX2 Hmx2	H6 family homeobox 1, NKX5-2	NKL homeobox	GnRH neurons. EMBL Atlas	Genecards AnimalTFDB TFe
HMX3 Hmx3	H6 family homeobox 1, NKX5-1	NKL homeobox	GnRH neurons.	Genecards AnimalTFDB
NKX2-1 Nkx2.1	NK2 homeobox 1, TTF1, TEBP	NKL homeobox	Fetal & adult thyroid & lung (1). Hypothalamic development (ARC, VMN). EMBL Atlas	Genecards AnimalTFDB TFe
POU1F1 Pou1f1	POU class 1 homeobox 1, PIT1	POU-I homeobox	Pituitary (1). EMBL Atlas	Genecards AnimalTFDB TFe
POU3F2 Pou3f2	POU class 3 homeobox 2, BRN2, OCT7	POU-III homeobox	General (1). Neuronal differentiation. EMBL Atlas	Genecards AnimalTFDB TFe
HESX1 Hesx1	HESX homeobox 1, ANF	PRD homeobox	EMBL Atlas	Genecards AnimalTFDB TFe
OTP Otp	Orthopedia homeobox	PRD homeobox	Hypothalamus: essential for development. Neuroendocrine cancers. EMBL Atlas	Genecards AnimalTFDB
PAX4 Pax4	Paired box 4, KPD	PRD homeobox	Pancreatic islet development and insulin secretion, diurnally expressed in pineal gland to antagonize PAX6. EMBL Atlas	Genecards AnimalTFDB TFe
PAX6 Pax6	Paired box 6, AN2	PRD homeobox	Developing hypothalamus GnRH. EMBL Atlas	Genecards AnimalTFDB TFe

PITX1 Pitx1	Paired-like homeodomain 1, BFT	PRD homeobox	Pituitary, tongue (1). EMBL Atlas	Genecards AnimalTFDB TFe
PITX2 Pitx2	Paired-like homeodomain 1, RIEG1, RGS	PRD homeobox	General (1). EMBL Atlas	Genecards AnimalTFDB
PROP1 Prop1	Prophet of Pit1, CPHD2	PRD homeobox	EMBL Atlas	Genecards AnimalTFDB TFe
GATA2 Gata2	GATA binding protein 2, NFE1B	Zinc Finger	Placenta, prostate (1). EMBL Atlas	Genecards AnimalTFDB TFe
IKZF1 Ikzf1	Ikaros 1, IK1, ZNFN1A1	Zinc Finger	General (1). Fetal and adult hemo-lymphopoietic system, anterior pituitary, hypothalamic neurons. EMBL Atlas	Genecards AnimalTFDB TFe
INSM1 Insm1	Insulinoma associated 1, IA-1	Zinc Finger	Fetal brain, pituitary (1). Developing endocrine tissues, neuroendocrine tumors. EMBL Atlas	Genecards AnimalTFDB TFe
REST Rest	RE-1 silencing transcription factor, Neural-restrictive silencing factor, NRSF, XBR	Zinc Finger	General (1). Neuronal progenitors and non-neuronal cells. Reduced expression or truncated variants in neurons and neuroendocrine cells. EMBL Atlas	Genecards AnimalTFDB TFe
SCRT1 Scrt1	Scratch 1, ZNF898	Zinc Finger	Neuronal differentiation, neuroendocrine cells of lung and lung cancers. EMBL Atlas	Genecards AnimalTFDB TFe

SP1 Sp1	Specificity protein 1, TSFP1	Zinc Finger	EMBL Atlas	Genecards AnimalTFDB TFe
FOXA2 Foxa2	Forkhead box A2, HNF3B	Forkhead	Embryonic development, establishment of tissue-specific gene expression and regulation of gene expression in differentiated tissues. Neuroendocrine tumors including prostate cancer. EMBL Atlas	Genecards AnimalTFDB TFe
SOX3 Sox3	SRY-box 3, PHP, MRGH	HMG box	Required during formation of hypothalamic-pituitary axis. EMBL Atlas	Genecards AnimalTFDB TFe
SOX10 Sox10	SRY-box 10, PCWH, WS4	HMG box	Salivary gland, spinal cord, trachea, whole brain (1). Neural crest and peripheral nervous system development. EMBL Atlas	Genecards AnimalTFDB TFe
ASCL1 Ascl1	Achaete-scute complex homolg 1, HASH1, MASH1	bHLH	Fetal brain, spinal cord, thymus, whole blood (1). Neuronal commitment, hypothalamic neuroendocrine differentiation, generation of olfactory and autonomic neurons. EMBL Atlas	Genecards AnimalTFDB TFe
NEUROD1 Neurod1	Neurogenic differentiation 1, BETA2, bHLHA3	bHLH	Differentiation: early retinal ganglion, inner ear sensory neurons, granule cells in hippocampus, endocrine pancreas, enteroendocrine small	Genecards AnimalTFDB TFe

			intestine, anterior pituitary corticotrophs. Neuroendocrine tumors. EMBL Atlas	
USF1 Usf1	Upstream transcription factor 1, bHLHb11, HYPLIP1, FCHL, MLTF	bHLH	General. EMBL Atlas	Genecards AnimalTFDB TFe
USF2 Usf2	Upstream transcription factor 2, c-fos interacting, bHLHB12, FIP	bHLH	General (1). EMBL Atlas	Genecards AnimalTFDB TFe
ARNT2 Arnt2	Aryl hydrocarbon receptor nuclear translocator 2, bHLHE1	bHLH-PAS	Fetal brain, spinal cord, whole brain (1). Essential hypothalamus development. EMBL Atlas	Genecards AnimalTFDB TFe
ARNTL Arntl	Aryl hydrocarbon receptor nuclear translocator like, BMAL1, MOP3	bHLH-PAS	General (1). EMBL Atlas	Genecards AnimalTFDB TFe
CLOCK Clock	Clock circadian regulator, bHLHE8, KAT13D	bHLH-PAS	General (1). EMBL Atlas	Genecards AnimalTFDB TFe
HIF1A Hif1a	Hypoxia inducible factor alpha subunit, bHLHE78, MOP1	bHLH-PAS	Smooth muscle (1). EMBL Atlas	Genecards AnimalTFDB TFe
SIM1 Sim1	Single minded homolog 1, bHLHE14	bHLH-PAS	Essential hypothalamus development. EMBL Atlas	Genecards AnimalTFDB
TBX19 Tbx19	T-box protein 19, TBS, TPIT	T-box	Pituitary (1). EMBL Atlas	Genecards AnimalTFDB TFe

Extended Table 1. Examples of sequence-specific transcription factors associated with regulation of neuroendocrine phenotype.

Links are provided to the pages for each transcription factor at: Genecards (repository of data for human gene and protein with links to many other databases), Animal TFBD (human or mouse database of transcription factor data) and Transcription Factor Encyclopedia (TFe, minireviews of human or mouse TFs that are currently in progress). Tissue distribution data is taken from (1) Vaquerizas et *al.* 2009, or the general literature, with links provided to the relevant EMBL Expression Atlas page.

1. Vaquerizas JM, Kummerfeld SK, Teichmann SA, Luscombe NM. A census of human transcription factors: function, expression and evolution. Nature reviews Genetics. 2009;10:252-63.

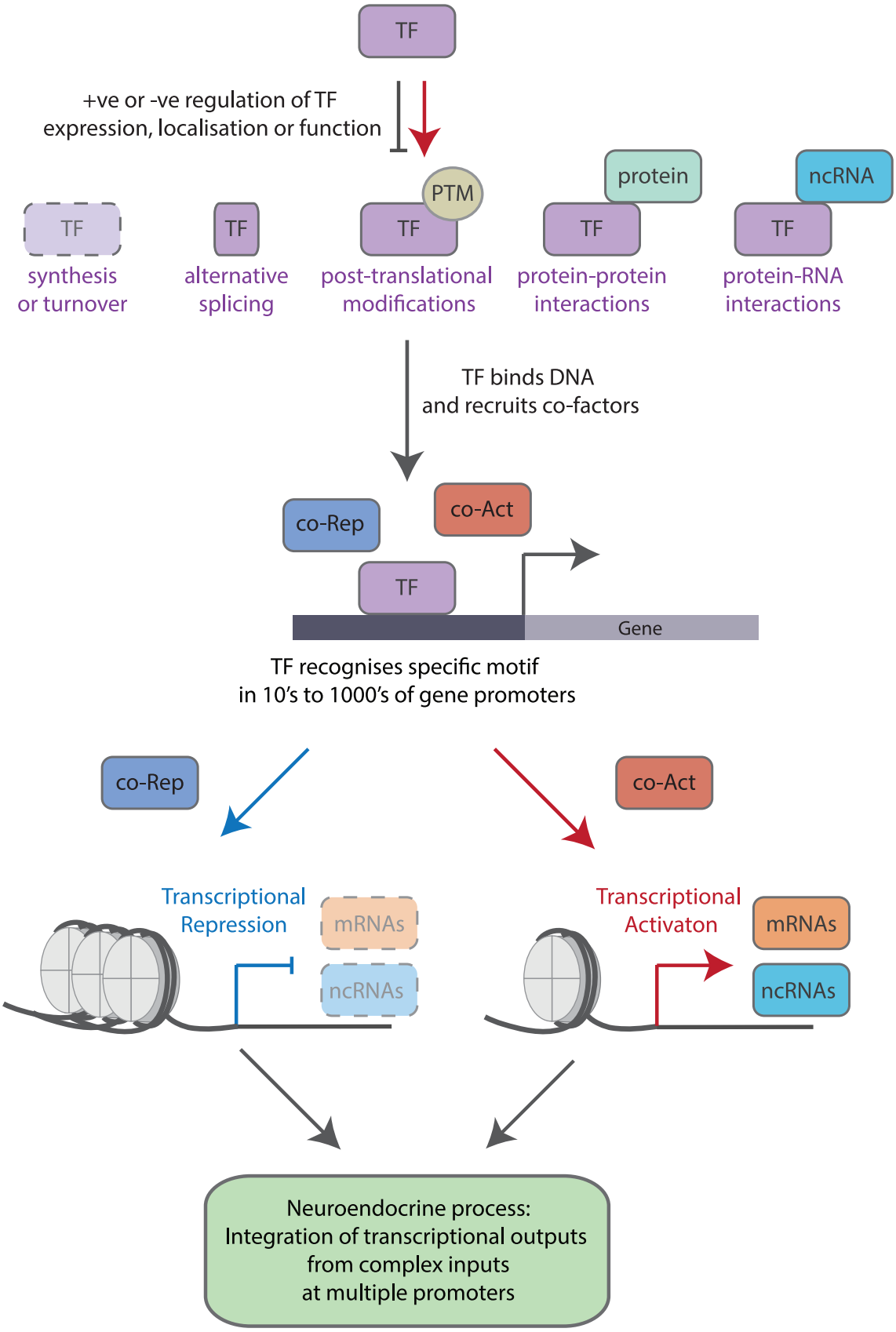

Figure 1. Key concepts: generalized pathway by which sequence-specific transcription factors direct physiological processes.

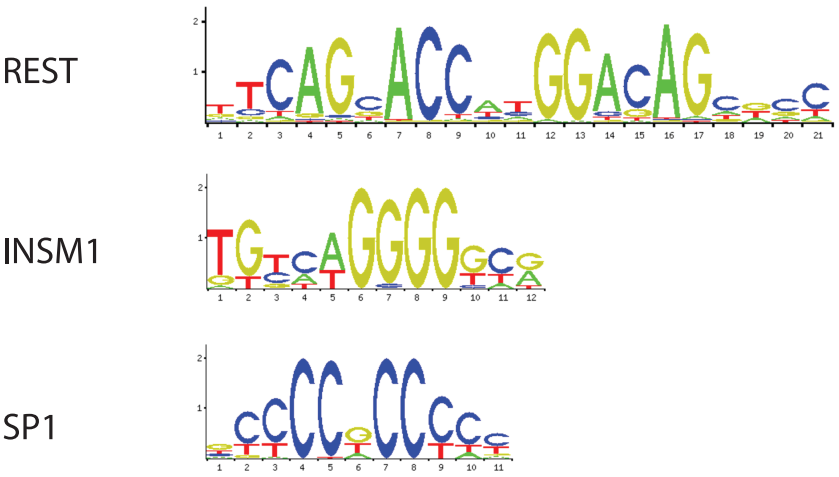

Figure 2. Examples of binding motifs for neuroendocrine-associated zinc finger TFs.

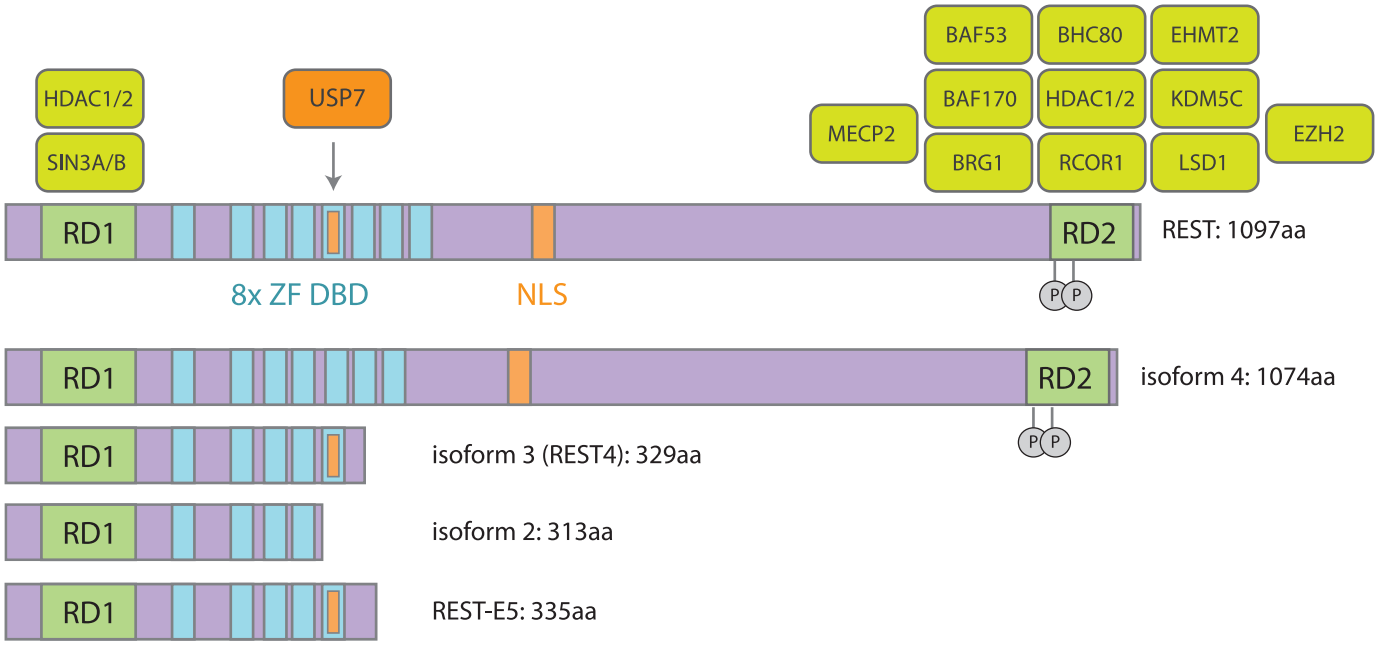

Figure 3. REST isoforms and co-factors.

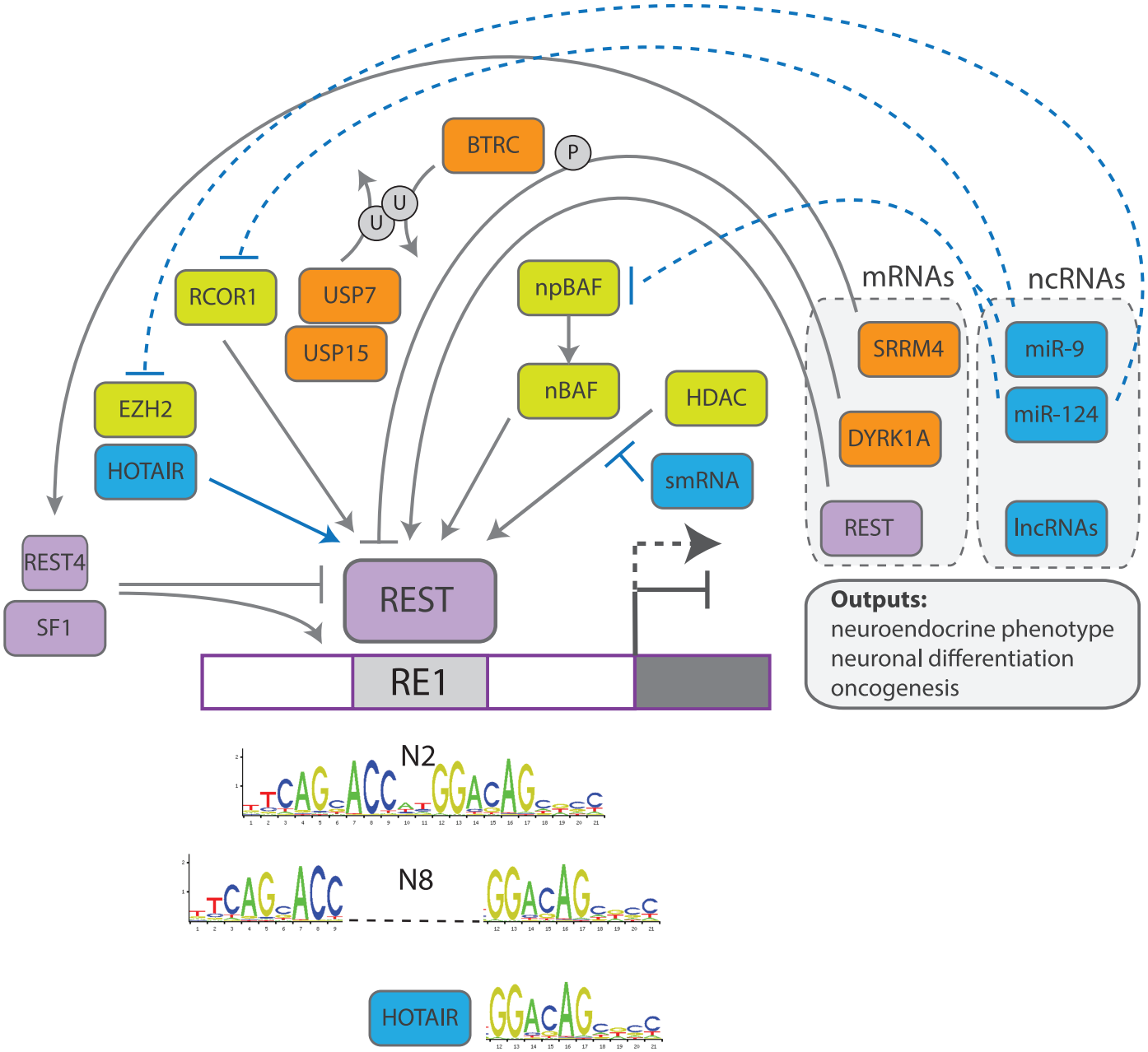

Figure 4. REST as a paradigm for diversity and feedback in TF regulation and function.

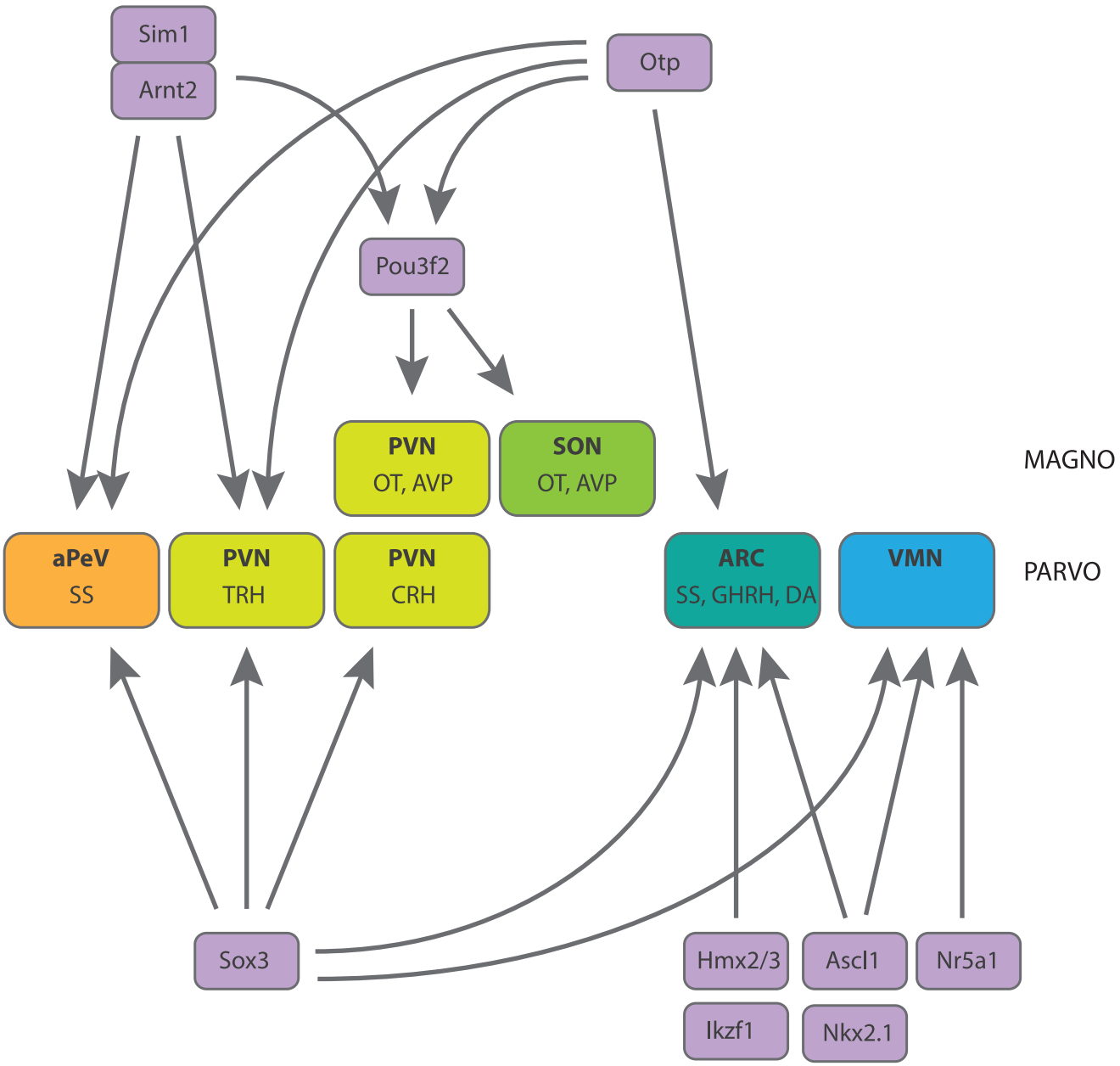

Figure 5. TFs required for development of the neuroendocrine hypothalamus.

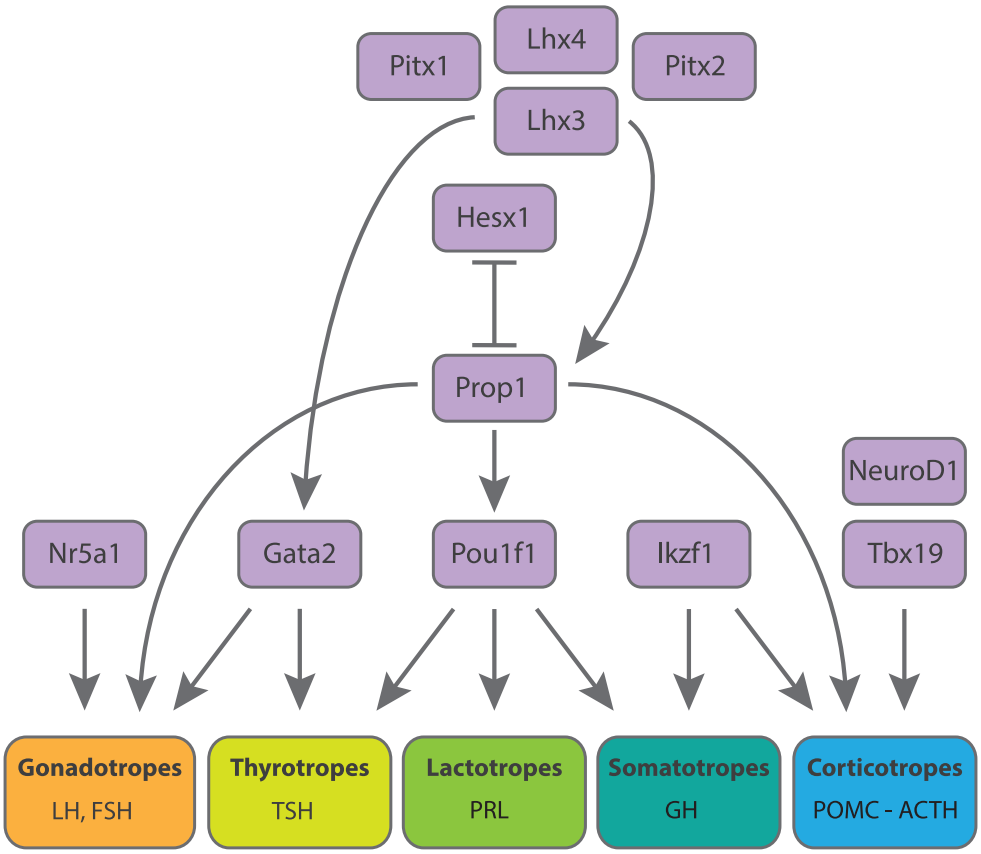

Figure 6. TF cascades in anterior pituitary development.

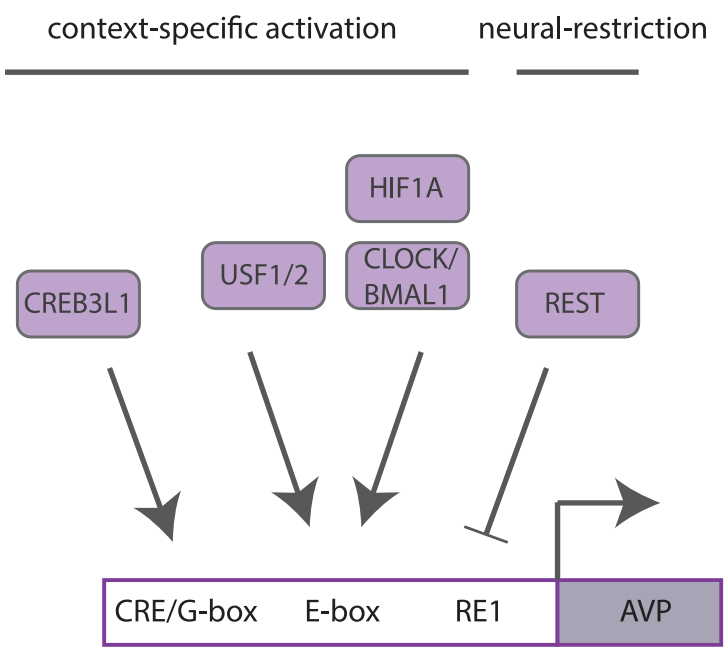

Figure 7. Context-dependent TF regulation of the AVP promoter.

Zinc finger GATA2 IKZF1 INSM1 REST SCRT1 SP1	***Basic leucine zipper*** ATF1 CREB1 (CREB) CREB3L FOS JUN
Homeobox ***LIM*** LHX3 LHX4 ***NKL*** HMX2 HMX3 NKX2-1 (TTF1) ***POU*** POU1F1 (PIT1) POU3F2 (BRN2) ***PRD*** HESX1 OTP PAX4 PAX6 PITX1 PITX2 PROP1	***bHLH*** ASCL1 (HASH1) NEUROD1 USF1 USF2 ***bHLH-PAS*** ARNT2 CLOCK HIF1A SIM1
	Nuclear receptor NR3C1 (GR) NR5A1 (SF1)
	HMG-box SOX3 SOX10
	Forkhead FOXA2
	T-box TBX19 (TPIT)

Table 1. Examples of sequence-specific transcription factors associated with regulation of neuroendocrine phenotype.