

From DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
Karolinska Institutet, Stockholm, Sweden

**INFERRING TRANSCRIPTIONAL REGULATION ON THE
PROMOTER LEVEL AND ITS APPLICATIONS TO
DISEASES**

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ABSTRACT

Gene regulation is important in maintaining cell identity and in higher organisms is a very complex process with many layers of regulation. Genome-wide transcriptional studies that define gene expression across different cells and tissues give important insights into overall gene regulation of a cell as well as the impact of dysregulation in diseases. With the recent advances of high-throughput sequencing methods, it has become increasingly feasible to elucidate transcriptional regulation in the cell, under normal conditions or during cell perturbation.

The aim of this thesis is, using these genome-wide profiling methods, to study in depth the regulatory promoter regions.

In Paper I, we knocked down 4 transcription factors in the THP-1 cell line and applied Cap Analysis of Gene Expression (CAGE) with sequencing. We were able to elucidate *de-novo* the transcriptional binding motifs of these 4 transcription factors as well as build perturbation driven gene regulatory networks. In Paper II, we utilized a similar approach on DYX1C1, a dyslexia susceptibility gene. Using perturbation studies and gene expression profiling with microarrays, the perturbed genes corresponded to the previously described neuronal migration phenotype that was speculated to be linked to the function of this gene. Furthermore, using mass spectrometry, we were able to identify novel protein interacting partners for DYX1C1 and combining with already available data build protein level interaction network. In Paper III, relying on the post-mortem brain samples from the FANTOM5 project, and using CAGE in conjunction with a single molecule sequencer, we identified brain specific transcriptional start sites and brain specific alternative promoters. Additionally, we identified differences between adult and infant brain, interestingly noting many of them originating from alternative promoters. We also classified differences between 15 brain regions into 4 distinct groups and built underlying transcription factor interaction networks. In Paper IV, using the FANTOM5 database we investigated the promoter structure and expression of 3 genes implicated in Rett syndrome. We identified novel promoters, silencing of FOXG1 in the cerebellum, as well as the low correlation between MECP2 and FOXG1 expression. Interestingly, although expression of FOXG1 is limited to the brain and MECP2 is ubiquitous, MECP2 motif activity is significantly lower in the brain than in other tissues, while no differences were observed for FOXG1 motif activity.

In summary, our genome-wide studies employing quantitative gene expression measures on promoter level resolution let us describe how cells are different, let us obtain insights into likely underlying regulatory mechanisms as well as gave us the opportunity to explore diseases.

LIST OF PUBLICATIONS

- I. **Morana Vitezić***, Timo Lassmann*, Alistair R. R. Forrest, Masanori Suzuki, Yasuhiro Tomaru, Jun Kawai, Piero Carninci, Harukazu Suzuki, Yoshihide Hayashizaki and Carsten O. Daub.
Building promoter aware transcriptional regulatory networks using siRNA perturbation and deepCAGE.
Nucleic Acids Res. 2010 Dec;38(22):8141-8.
*Authors contributed equally

- II. Kristiina Tammimies, **Morana Vitezić**, Hans Matsson, Sylvie Le Guyader, Thomas R. Bürglin, Tiina Öhman, Staffan Strömblad, Carsten O. Daub, Tuula A. Nyman, Juha Kere, and Isabel Tapia-Páez.
Molecular networks of DYX1C1 gene show connection to neuronal migration genes and cytoskeletal proteins.
Biol Psychiatry. 2013 Mar 15;73(6):583-90.

- III. Francescato Margherita*, **Morana Vitezić***, Patrizia Rizzu, Javier Simón-Sánchez, Robin Andersson, Hideya Kawaji, Masayoshi Itoh, Naoto Kondo, Timo Lassmann, Jun Kawai, Harukazu Suzuki, Yoshihide Hayashizaki, Carsten O Daub, Albin Sandelin, Michiel JL de Hoon, Piero Carninci, Alistair RR Forrest and Peter Heutink.
A high resolution spatial-temporal promoterome of the human brain.
Manuscript.
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- IV. **Morana Vitezić**, Michiel JL de Hoon, Nicolas Bertin, Robin Andersson, Leonard Lipovich, Timo Lassmann, Albin Sandelin, Alistair R R Forrest, Piero Carninci, Alka Saxena and the FANTOM Consortium.
FANTOM5 reveals the genomic architecture of the genes implicated in Rett Syndrome.
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ADDITIONAL PUBLICATIONS

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Linking promoters to functional transcripts in small samples with nanoCAGE and CAGEscan.

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- II. Luba M. Pardo*, Patrizia Rizzu*, Margherita Francescato, **Morana Vitezic**, Gwenaël G.R. Leday, Javier Simon Sanchez, Abdullah Khamis, Hazuki Takahashi, Wilma D.J. van de Berg, Yulia A. Medvedeva, Mark A. van de Wiel, Carsten O. Daub, Piero Carninci and Peter Heutink.

Regional differences in gene expression and promoter usage in aged human brains.

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LIST OF ABBREVIATIONS

A	adenin
bp	base pair
C	cytosine
CAGE	Cap Analysis Gene Expression
cDNA	complementary DNA
CDS	coding sequence
CGI	CG island
ChIP	Chromatin Immunoprecipitation
DNA	deoxyribonucleic acid
DYX1C1	dyslexia susceptibility 1 candidate gene 1
DPE	downstream promoter element
ENCODE	Encyclopedia of DNA Elements
FANTOM	Functional Annotation of the Mammalian Genome
G	guanine
GO	gene ontology
Inr	initiator element
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	liquid chromatography tandem mass spectrometry
lncRNA	long non-coding RNA
miRNA	micro RNA
mRNA	messenger RNA
PIC	preinitiation complex
PolII	RNA Polymerase II
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
siRNA	small interfering RNA
T	threonine
TBP	TATA binding protein
TF	transcription factor
TFBS	transcription factor binding site

tRNA	transfer RNA
TSS	transcription start site
U	uracil
UTR	un-translated region

1 BACKGROUND

The year 2001 gave the first draft of the human genome, a first view at the whole collection of all the nucleotides, letters and their order that make up our human DNA (Lander *et al*, 2001; Venter *et al*, 2001). This enormous project conducted in big consortia around the world showed that the number of genes we possess is much less than was previously believed, around 30,000 out of the earlier predicted 100,000 (Bork P. and Copley R. 2001). Yet, these genes and their inner workings give rise to over 200 different cells that make up our body (Vickaryous and Hall, 2006), all containing the same DNA sequence, but with a broad array of functions due to genes being regulated in different ways. Gene regulation is important in maintaining cell identity and in higher organisms is a very complex process with many layers of regulation. Genome-wide transcriptional studies that define gene expression across different cells and tissues give important insights into overall gene regulation of a cell as well as the impact of dysregulation on diseases.

2 RNA AND TRANSCRIPTIONAL REGULATION

2.1 FROM DNA TO RNA – TRANSCRIPTION

Inside the nucleus each of our cells contains the complete hereditary material, the set of 46 chromosomes that we inherited from our parents, half from the mother and half from the father. The chromosomes are made up of DNA helixes wrapped around histone proteins. DNA is double stranded and DNA length is measured in base pairs (bp). Basic building blocks of the DNA are nucleotides, the purines adenine (A) and guanine (G) together with the pyrimidines cytosine (C) and thymine (T), making pairs to connect the backbones of the two DNA strands (adenine with thymine and guanine with cytosine).

Genes are stretches on the DNA that define heritable, functional units. The entirety of heredity information of an organism forms its genome. DNA is like a blueprint and for a gene to be used, it needs to be transcribed from DNA into RNA, meaning that its sequence is copied. RNA is, like DNA, a nucleic acid, but it uses the nucleotide uracil instead of thymine and in the cell it occurs mostly in single stranded form, unlike DNA which is double-stranded. The central dogma of molecular biology (Crick F, 1970.) describes the information flow from DNA is part of the basic information flow in the cell that defines that DNA transcribes into RNA which in turn gets translated into proteins.

2.2 RNA POLYMERASE

Transcription is performed by RNA polymerase, an enzyme that uses the DNA strand as a template to build RNA base by base by moving along the DNA strand. The products of RNA polymerase are: messenger RNA (mRNA) that is the template for translation into proteins as well as non-coding RNA that is a wide group of genes that do not get translated into proteins but have other, mostly functional (such as transfer RNAs or ribosomal RNAs) or regulatory functions (such as micro RNAs). Non-coding RNA includes transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNA (miRNA), short RNAs and long non-coding RNAs (lncRNAs). Humans have several different types of RNA polymerases: polymerase I synthesizes rRNA, polymerase II synthesizes mRNA and most snoRNA and miRNA and polymerase III synthesizes tRNAs, rRNAs and other small RNAs.

Polymerase II (PolII) is the most studied one and due to its complex level of control, requires many additional binding factors. For PolII transcription to function properly, additional proteins are recruited to the DNA strand to form the transcription preinitiation complex (PIC) together with the polymerase. The role of the PIC is positioning the polymerase over the transcription start site (TSS), the position from where the transcription of the gene starts.

2.3 TRANSCRIPTION STARTS AT THE PROMOTER REGION

The PIC binds at the promoter, a specific position on the DNA where transcription is initiated. The promoter is an important part of the gene, without the promoter transcription of the gene cannot be initiated and no gene product is obtained. The promoter is located upstream of the gene and is usually between 100 and 1000 bp long. Depending on distance to the TSS and the specific function, we can classify the promoter into three parts: the core promoter in close proximity upstream of the TSS; the proximal promoter region that comprises about 300 bp upstream from the TSS and includes specific regulatory elements binding sites, most notably for transcription factors; the distal promoter region up to several 10 kb upstream of the TSS contains enhancers or insulators.

The core promoter region, except the TSS itself, contains binding sites for the PIC, RNA polymerase and general binding sites. These include the TATA box (Lifton *et al*, 1978), a sequence that contains a TA-rich pattern about 30bp upstream from the TSS and binds the TATA binding protein (TBP) involved in DNA strand separation during the process of transcription and part of the PIC. Only about 10-20% of all promoters have a clear TATA box motif (Valen and Sandelin, 2011) and are linked to genes expressed in specific tissues (Carninci *et al*, 2006). Other core promoter elements include the initiator element (Inr), independent of the TATA box but can also initiate transcription on its own (Smale and Kadonga, 2003), the downstream promoter element (DPE), the TFIIB recognition element (BRE) and the CpG island (CGI).

Between 40% and 70% of human promoters contain a CGI (Sandelin *et al*, 2007), meaning there are more CG stretches than expect for the local number of C and G nucleotides (Deaton and Bird, 2011). Unlike TATA-box connected proteins, CGIs are most often associated to ubiquitously regulated genes.

Comparison of the TSS distribution of different genes suggested that promoters can be roughly classified into ‘sharp’ and ‘broad’ according to the spread of their distribution across the nucleotides, where the sharp class often corresponds to tissue specific promoters with TATA boxes. The broad promoter class has an over-representation of CGIs usually active in many tissues (Carninci *et al*, 2006). Recent an additional promoter type was suggested that includes differentially regulated genes, often regulators in multicellular development and differentiation that contain large CGI stretches (Lenhard *et al*, 2012).

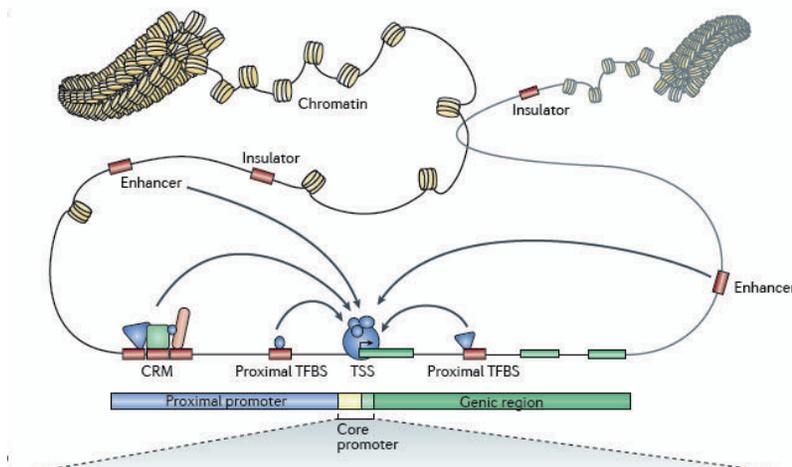


Figure 1 A view of the promoter region and its regulatory elements. *Cis*-regulatory modules (CRM) are clusters of TFBSs. Adjusted from Lenhard *et al*,2012 Used with permission from the Nature Publishing Group.

The majority of eukaryote promoters are comprised not only of a single TSS, but contain several TSSs spread over a region covering 30–100 nt or more (Sandelin *et al*, 2007). TSS distribution for a given promoter is often conserved between species (Frith *et al*, 2006).

2.4 MRNA AS THE RESULT OF THE TRANSCRIPTION

The direct result of transcription is precursor mRNA that requires further processing before it becomes functional. On its “front”, the 5' end, it gets an addition in the form of a cap, a modified guanine nucleotide, shortly after the start of transcription by the cap-synthesizing complex associated with RNA polymerase. The 5' cap consists of a terminal 7-methylguanosine residue that is linked through a 5'-5'-triphosphate bond to the first transcribed nucleotide. Another processing step is splicing, the removal of inactive parts, introns, that have no coding information and leaving in the protein-coding parts, exons. Sometimes precursor mRNAs arising from the same gene can be

spliced in different ways, giving rise to different proteins in the process called alternative splicing. Another possible process is editing, where nucleotides directly in the mRNA are changed and can result in an altered protein sequence in the case of protein coding RNA. The final processing step happening at the 3' end is called polyadenylation, an addition of a 'tail' of adenosine residues (poly(A) tail) added to the free 3' end. This reaction is catalyzed by polyadenylate polymerase. Similar to alternative splicing, there can be more than one polyadenylation variant of one mRNA. The purpose of the poly(A) tail is to protect mRNA from degradation by exonucleases, for export of the mRNA from the nucleus as well as for loading into the ribosome for translation.

A mature, fully processed mRNA is thus composed of the cap at the 5' end, followed by the 5' untranslated region (UTR) that contains regulatory regions, the coding sequence (CDS) that includes the code for the start and stop codons used in translating the mRNA into proteins, the 3'UTR and at the end the poly(A) tail.

2.5 TRANSCRIPTION FACTORS ARE THE MAIN REGULATORY ELEMENTS

The regulatory elements that bind at the promoter sites are specific proteins known as transcription factors (TFs). A TF can act alone or with other proteins in a complex by promoting (activation) or blocking (repression) the recruitment of RNA polymerase to the specific promoter (Lee and Young, 2000). However, regulation of gene expression in eukaryotes is highly complex and depends on sets of TFs rather than individual TFs (Elkon *et al*, 2003). The main trait that makes a protein a transcription factor is having one or more DNA binding domains that recognize and bind to specific sequences of DNA in the promoter region. One or more DNA-binding domains are often part of a larger protein consisting of additional domains with differing function. The additional domains often regulate the activity of the DNA-binding domain.

There are approximately 1,400 proteins in the human genome that contain DNA binding domains and most of these are presumed to function as TFs (Vaquerizas *et al*, 2009). The TFs are the largest family of human proteins, comprised of about 10% of all coding genes. The promoter composition of the genes is one of the major determinants of gene regulation including multiple transcription binding sites that interact with a specific combination of transcription factors (TF). Eukaryotes achieve this diversity by combining a small number of transcription factors whose activities are modulated by

diverse sets of conditions. Different functionalities can be held by one TF by its association with different co-factors. These factors may act as global TFs that assist their gene-specific partners in their function, and may thus activate or repress transcription depending on the partner motif and the condition. Usually, several binding sites for distinct transcription factors are found around each gene and efficient expression requires the cooperative action of several different TFs (Pilpel *et al*, 2001). This way, combining 2,600 TFs can regulate the whole genome in an efficient way during development and maintenance (Brivanlou and Darnell, 2002).

The TF binds to its specific DNA sequence at the position called the transcription factor binding site (TFBS). Due to the weak interactions of such bonds, the TF can bind not just to one sequence but to a subset of closely related sequences, making the detection of such regions much more complicated. Because these TFBS tend to be short sequences, many potential binding sites can be found throughout the genome, not necessarily meaning all of them are active binding sites, additionally complicating the search for true active TFBS in a specific cell. Additionally, although we know most of these proteins to be TFs due to their binding domains, for a vast majority we do not know the sequence they bind to with confidence.

Many attempts have been made to identify the TFBSs, both experimentally and computationally. Computational methods include identifying the presence of a sequence motif in a set of DNA sequences known to bind the protein. The difficulty here is that even if the motif is present, we cannot know if the TF really binds there *in vivo*. The most efficient methods combine experimental efforts with computational analysis. Chromatin Immunoprecipitation (ChIP) is a technique where a protein of interest is selectively immunoprecipitated from a chromatin preparation to determine the DNA sequences associated with it (Collas 2010). The obtained sequences that bind that protein or TF can then be identified on a tiling microarray (ChIP-chip) or by sequencing (ChIP-Seq). To be able to perform experiments for specific transcription factors, however, specific antibodies are needed whose production is difficult. For many of the transcription factors antibodies are not yet available (Sikder and Kodadek, 2005). Additionally, computational analysis of such datasets is very demanding for example due to low resolution of the ChIP-chip method or the longer reads than binding sites in ChIP-Seq that can make it hard to determine the exact sequence the protein recognizes. Another emerging experimental technology to identify binding sites

in vitro is Systematic Evolution of Ligands by Exponential Enrichment (SELEX). This method uses rapid selection of nucleic acids (single- or double-stranded RNA or DNA), which have high affinity to a molecular target such as a TF (Oliphant *et al.*, 1989; Tuerk and Gold, 1990). Combining SELEX with next-generation sequencing has recently led to identifying over 400 TF binding motifs (Jolma *et al.* 2010; 2013). Over-selection can be a problem for SELEX technology that enriches for specific binding sequences since TFs *in vivo* bind to biologically important medium or low affinity TFBSs as well.

Due to their important roles in development, intercellular signaling and cell cycle, some human diseases have been associated with mutations in transcription factors. One such disease is the Rett syndrome (Paper IV), a neurodevelopmental syndrome linked to mutations in the MECP2 transcription factor.

Additional regulatory elements, such as distal acting enhancers and non-coding RNAs as well as epigenetic regulators (Chawan *et al.*, 2011) can influence the start of transcription and formation of a fully functional mRNA. To be able to characterize transcriptional events, different genome-wide techniques can be utilized to gain a better insight of the current transcription ongoing in the cell and the underlying regulation.

2.6 MEASURING DIFFERENCES IN GENE EXPRESSION

Genome-wide expression measurement of a diseased state together with a reference normal state allows identifying differences global level using differential expression analysis. In the first years when microarrays have been introduced, differentially expressed genes were inferred using a fixed threshold cut-off for expression differences, for example a two-fold increase or decrease in expression. Improved methods to infer significance were based on replicate measurements for ranking genes according to their possibilities of differential expression and selection of a cut-off value for rejecting the null-hypothesis that the gene is not differentially expressed (Leung and Cavalieri, 2003). Replication of an experiment is important to obtain the variation in the gene expression for statistics calculation, ideally every experiment performed in at least triplicates (Lee *et al.*, 2000). Replication can be either technical, by using the same RNA sample multiple times, or biological by extracting RNA from different samples. This is particularly important in tissue samples to characterize expression variability caused by variability of the tissue so that one sample of a tissue might not be a representative sample. Statistical methods such as Student's t-test, ANOVA, Bayesian

method, or Mann-Whitney test can be used to rank the genes from replicated data (Storey and Tibshirani, 2003). Setting a ad-hoc cut-off for differential expression is difficult, because of the false positives (Type I error) and the false negatives (Type II error). Furthermore, performing statistical tests for tens of thousands of genes creates a multiple hypothesis-testing problem. Therefore, it is necessary to control the false discovery rate (FDR) (Reiner *et al*, 2003), the expected proportion of false positives among the number of rejected hypotheses and base the cut-off on the samples that satisfy the FDR criteria.

3 RNA EXPERIMENTS FOR GENOME WIDE STUDIES

There is a range of technologies that target the mRNA in a specific way, allowing us to capture the expression of a certain cell line or tissue.

For the 4 different papers, we applied a set of different techniques: RNA perturbation in papers I and II as well as CAGE in papers I, III and IV.

3.1 RNA PERTURBATION STUDIES

Many approaches aim at understanding the interactions between genes that ultimately govern phenotype and disease pathology (Birney *et al*, 2007). The complex interactions among transcription factors derived from such networks point to diverse regulatory programs responsible for cell differentiation during development and cellular responses to outside stimuli. A powerful technique to understand gene regulatory networks is the perturbation of individual transcription factors in concert with high-throughput profiling of all genes to assess the impact. Perturbation can be performed by either knocking down individual genes or up-regulation. RNA interference (RNAi) uses small RNA molecules to inhibit gene expression, typically by causing the destruction of specific mRNA molecule and thus knocking-down the gene expression. This method employs the cell's own RNAi pathway in which small interfering RNAs (siRNA) are used as a template to mark the target mRNA for cleavage (Voorhoeve and Agami, 2003). Alternatively, overexpression of a gene can be induced by putting the gene and its promoter into a plasmid construct inserted into the cell.

3.2 CAP ANALYSIS GENE EXPRESSION (CAGE)

Capturing the exact transcription amount and positions of the TSS in the cell is an important goal for genome-wide expression studies. Cap-analysis gene expression (CAGE) captures the 5'-end of the mRNAs in the cell (Shiraki *et al*, 2003; Kodzius *et al*, 2006). The strength of CAGE is to comprehensively map the vast majority of human transcription starting sites and hence their promoters, and simultaneously decipher the expression of the RNAs produced at each promoter. Thus, CAGE allows for high- throughput gene expression profiling with simultaneous identification of the tissue/cell/condition-specific TSSs, including promoter usage analysis and determination of the expression level at each promoter (Takahashi *et al*, 2012).

Precisely mapping the TSS position allows for identifying regulatory elements, such as core and proximal promoters and the TFBSs that are responsible for transcription. Bioinformatic analysis allows for selection of promoters having similar expression profiles that are analyzed for the presence of common TFBS. Coupled to the determination of the expression of transcription factors, which drive the gene transcription, this analysis allows reconstructing the regulatory networks that drive gene expression (Suzuki *et al*, 2009). Expression is represented by the number of CAGE 'tags' mapping to a certain TSS. By counting the number of CAGE tags for each TSS within a gene, we can determine not only the RNA expression level on a digital resolution but also the various alternative promoters being used, allowing comprehensive mapping of promoters in mammalian genomes (Carninci *et al*, 2006).

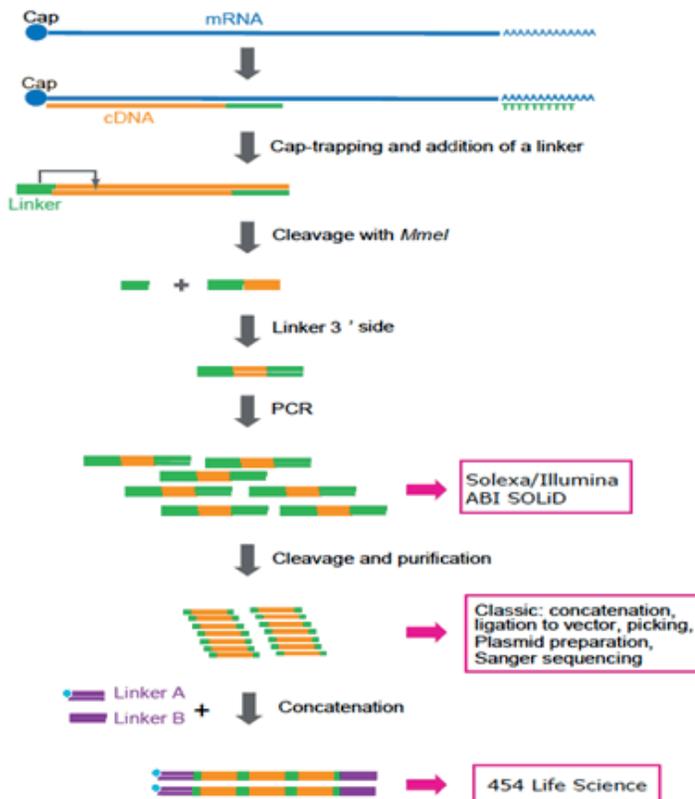


Figure 2 Representation of CAGE preparation protocol adapted to various platforms.

Illustration from <http://www.osc.riken.jp/english/activity/cage/basic/>

A CAGE library is produced in the following steps. First, cDNA complementary strands are synthesized from total RNA extracted from cells or tissues by using random or oligo dT primers. The 5' end of cDNA is then selected by using the cap-trapper

method. Second, a biotinylated linker is attached to 5' end of single-strand cDNA acquired by removing the RNA strand using RNaseI. This linker contains recognition sites that are essential for cloning, short specific base sequences, and endonuclease recognition sites (MmeI or EcoP15I). After the second cDNA strand is synthesized, 20 nucleotides (MmeI) or 27 nucleotides (EcoP15I) are cut from the 5' end to make the CAGE tag. Next, a linker is attached to the 3' side of the tag sequence to amplify it. Previously, fragments were cleaved and concatenated in CAGE tags, but current next generation sequencers (see below) do not require cleavage and the tags can be directly sequenced to produce millions of tags per sample (Takahashi *et al*, 2012). This next-generation sequencer adapted CAGE is often referred to as deepCAGE.

CAGE was also adapted for the single molecule sequencer Heliscope. The changes in this simplified protocol include generation of only first strand cDNA using an excess of random primer. The capped end is captured on magnetic streptavidin beads. Released first-strand cDNA is poly(A)-tailed and blocked and then loaded directly onto the HeliScope flow cell for sequencing (Kanamori-Katayama *et al*, 2011).

4 GENOME WIDE EXPRESSION PROFILING METHODS

4.1 MICROARRAYS

Studies of gene expression were previously possible on only one or a few genes at a time. The ability to perform genome expression profiling on the whole set of genes in a cell came in the mid 1990s with the advent of expression microarray technology (Schena *et al*, 1995). This technology allows for the study of thousands of transcripts at the same time. The technology is based on a set of probes immobilized on a glass slide in the form of spots. Each spot contains picomoles of a specific probe (either a part of a gene or some other specific sequence) and it uses the mechanism of hybridization to connect the DNA or RNA target strands to the probes. The unhybridized targets are washed away and the hybridization of the probe and its target is then detected by fluorophore or chemiluminescence labeled targets to determine relative abundance of nucleic acid sequences in the target. Total strength of the signal on one spot depends on the amount of targets binding to the probes on that spot. The signal then needs to be normalized, usually using the background probe levels. The expression levels can be determined using relative normalization in the comparison of an experiment and its control for each spot. Thus the values are only explainable in relative conditions and are not a direct measurement of the level of expression of the target

The most popular genome expression technologies include in-situ-synthesized arrays and high-density bead arrays. In situ-synthesized arrays are high-density oligonucleotide probe microarrays (such as Affymetrix GeneChip). They are made using photolithography, using light to create a pattern. The method relies on UV masking and light-directed combinatorial chemical synthesis on a solid support to selectively synthesize probes directly on the surface of the array, one nucleotide at a time per spot, for many spots simultaneously. The probes are 25 bp long. The other popular high-density methods are BeadArrays manufactured by Illumina. This technology is based on color-coded 3-micron silica beads that randomly self assemble in either a fiber-optic bundle substrate that then themselves assemble into arrays, or a silica slide substrate. When randomly assembled on one of these two substrates, the beads have a uniform spacing of approximately 5.7 microns, with a packing density of

about 40,000 array elements per square millimeter. This gives the Bead Array platform about 400 times the information density of a typical spotted array. Each bead is covered with hundreds of thousands of copies of a specific oligonucleotide that act as the capture sequences in one of Illumina's assays. The sequence specific probe is 50 bp long (Miller and Tang 2009). Illumina BeadArrays were used in Paper II.

The microarrays are usually genome based (containing probes for about 47,000 different RNAs) and the probes are usually based on the 3' end of the expressed mRNA since they target the poly-A tail of mRNAs and contain probes that are clustered for detection of sequences at the 3'-end of the target. While these expression arrays can report changes in transcript abundance, they are only able to distinguish transcripts of the same gene if the transcripts differ in their last few transcribed exons. Since microarrays contain many thousands of probes, there is the possibility of cross-reactivity of samples to different genes giving then a false estimation of gene expression annotated for that probe. Many changes in transcript expression are caused by inclusion of alternate exons or alternative start sites, which would produce different isoforms of the gene that are not visible to 3' expression arrays. To address the challenge of alternative splicing that produces alternative exon expression, several splice-sensitive microarray platforms have been developed.

Studying gene expression using microarrays has had big impacts on medical research since this technology can compare expression of gene based probes between diseased and normal samples, cancers and tissues, enabling the identification of potential targets for treatments (van t'Veer *et al*, 2002). This technology has fundamentally altered biology and medicine by allowing the study of expression patterns across an entire genome.

4.2 NEXT-GENERATION SEQUENCING – HISTORY AND OVERVIEW

Sequencing is a method that determines the order of the nucleotides in a sequence. One of the first established methods for sequencing was Sanger sequencing, based on chain termination. In this method, an extension is initiated at a specific site on the template DNA by using a short oligonucleotide primer complementary to the template at that region. Included with the primer and polymerase are the four bases, along with a low concentration of a chain terminating nucleotide (most commonly a di-deoxynucleotide). Incorporation of the chain terminating nucleotide results in a series of related DNA fragments that are terminated only at positions where this particular

nucleotide is used. The fragments are then size-separated by electrophoresis in a slab polyacrylamide gel or a glass capillary (Sanger *et al*, 1977).

The next steps to improve this technology came with the development of different colored terminating nucleotides to enable the reaction to run in one tube (Smith *et al.*, 1985) as well as the development of machines enabling many reactions to run at the same time thus automating the process (Smith *et al*, 1986). These were all prerequisites for one remarkable project of mapping the whole human genome. In the works since the mid 80s, the Human Genome Project (HGP) officially started in 1990 and finished in 2003. The project was also marked with a huge investment in genome sciences, which focused on parallelization and automation of sequencing methods.

The next big brake for sequencing came in 1996 with establishing of pyrosequencing (Ronaghi *et al*, 1996), a method very different from previous ones. It is based on the incorporation of nucleotides one at a time as a complementary, single strand to the single stranded DNA template. As the nucleotides are added, light from the release of PPi is emitted and measured (usually via a CCD camera). The addition of each nucleotide is controlled, allowing for easy tracking of the desired sequence loci.

Different methods as well as automation, massive parallelization of processes and lowering of sequencing costs prepared the market for the advent of sequencing machines.

4.2.1 The Roche 454

The new era of next-generation sequencing was ushered in with the release of the first next-generation sequencer, Life Sciences 454 (Margulies *et al*, 2005). For a sample to be sequenced, it needs to be randomly cut into smaller pieces, adding adapter sequences to the ends, then combining the fragments with Sepharose beads (diameter ~28 µm) which have been coated with oligonucleotides complementary to the adapters. The sample is mixed with an excess of beads so that most beads bind only a single template molecule. The beads with the bound DNA are subjected to emulsion PCR (Nakano *et al*, 2003), which amplifies the DNA templates from a single copy to approximately 10 million copies on each bead. Subsequently, the enriched, template-carrying beads are deposited into open wells arranged along one face of a 60×60 mm² fibre-optic. The wells are sized to fit only a single bead and each plate contains approximately two million wells. Reagents are supplied to the picotiter plate for sequential rounds of sequencing by synthesis using a modification of the pyrosequencing method. The chemiluminescent event is detected by a camera. The location of each template

molecule in its unique well of the 454 picotiter plate is recorded and computational assembly of the sequences of all templates happens simultaneously. The 454 sequencer is equipped with an integrated computer which allows for signal processing in real time. This system has very low base calling error rate due to only one nucleotide being added at a time but it has an issue when a template molecule contains multiple bases of the same type, such as a run of AAAA's, then multiple bases are synthesized onto the copy strand all at once, creating a larger emission of light. It is difficult for the system to accurately count the number of bases in homopolymers longer than eight or nine bases.

The first 454 produced approximately 500,000 sequences (or 25 Mb) of data, with reads 80-120 bases long. In 2012, an upgrade to the 'GS' system known as 'FLX+' increased the average read length to 700 bases for ~1 million reads or 3 Gb of data from a run. The cost per run remains about \$8,000. To lower the cost and increase yield, a multiplex strategy that involves both barcodes for individual samples, and a set of gaskets which divide the surface of the sequencing plate into sub-sections are employed. The advantage of this system is that it can deliver long reads and deep sequencing which is particularly suitable for de-novo sequence assembly. Roche 454 sequencer is used to sequence the data in Paper I.

4.2.2 Illumina Genome Analyzer

Around the same time of the release of the 454, a new technology was being developed at the University of Cambridge using the bridge-amplification technology or sequencing-by-synthesis. It was able to generate for the first time 1 Gb in a run. The instrument was called the Solexa 1G (Bentley *et al*, 2008).

Prepared libraries are sequenced on a flow cell, which has a lawn of two oligos complementary to the different adapter sequences. Cyclical reactions produce a 'cluster' of around 1000 copies of the original library molecule. Clusters are made single-stranded by cleaving of the adapter sequence. Hybridization of a sequencing primer is then followed by addition of fluorescent terminators in a cyclical reaction (similar to pyrosequencing, but using just one color). Nucleotides are incorporated by polymerase into the growing DNA strand. The flow cell is imaged to determine which nucleotide has been incorporated into each individual cluster. The terminator is removed by chemical cleavage ready for the next round of incorporation, imaging and cleavage.

In 2007, the early Solexa-based sequencers generated reads of 35 bp and generated around 30 M sequences (or 1 Gb) of data from a flowcell.

The newest version of this sequencer, the HiSeq, is currently the most widely used sequencer and can generate 2.4 billion sequences (300 Gb) of data in one run, with the read length of 100 bp as compared to the first read length of just 26 bp.

4.2.3 Life Sciences SOLiD and Ion Torrent

The third major technology to enter was the SOLiD, Sequencing by Oligo Ligation Detection, which uses sequencing-by-ligation. On a SOLiD flowcell, the libraries can be sequenced by 8 base-probe ligation which contains ligation site (the first base), cleavage site (the fifth base), and 4 different fluorescent dyes (linked to the last base) (Mardis E, 2008). The fluorescent signal is recorded while the probes are bound to the template strand and diminished by the cleavage of probes' last 3 bases.

Originally, the read length of SOLiD was 35 bp and the output was 3 Gb of data per run. The new generation has improved read length, accuracy, and data output of 85 bp, 99.99%, and 30 G per run, respectively (Lin L. *et al*, 2012). Despite its high accuracy, SOLiD's short read length makes it a less popular alternative to the Illumina Hi-Seq.

The newer system Ion-Torrent has a semiconductor base detection system. The sequencing itself is an approach similar to pyrosequencing but with a twist. It detects hydrogen ions that are released during the polymerisation of DNA, as opposed to the optical methods used in other sequencing systems. The sequencing is performed in the wells of a semiconductor chip into which individual emulsion PCR beads can be loaded. Sequencing is performed in the same cyclical manner but there are no additional enzymes and natural, rather than fluorescently modified, nucleotides are used. As each nucleotide is incorporated hydrogen ions are released, which change the pH of the solution in the well. The change in pH is detected by the chip which has an ion sensor at the bottom of each well reading out the data (Rotherberg *et al*. 2011). Ion Torrent produces similar amount of data to HiSeq but with a longer read length of up to 200bp.

Other vendors are competing with the current 'big three' of the next-generation sequencing, including Complete Genomics, which uses DNA nanoballs and unchained sequencing by ligation (Lee *et al*, 2010) but does all the sequencing 'in house' as a service without producing any machines.

Another angle is making the machines more approachable to general users, particularly aiming at clinicians by miniaturizing the products. Both Illumina and Ion Torrent have smaller versions, MiSeq and Ion PGM, that give smaller yields but are more affordable and aimed at personal genomics for sequencing few genes of many different individuals.

4.2.4 Heliscope single molecule sequencing

Helicos Biosciences sequencer Heliscope was the first sequencer that was truly single molecule based, meaning that it did not need any of the amplification steps that the other sequencers use. It uses DNA fragments with added poly-A tail adapters attached to the flow cell surface. The next steps involve extension-based sequencing with cyclic washes of the flow cell with fluorescently labeled nucleotides (one nucleotide type at a time, like with the early Sanger method). The reads are short, up to 55 bases per run, but allow for more accurate reads of stretches of one type of nucleotides (Thompson and Steinman, 2010). Heliscope was used for sequencing the data described in Paper III and Paper IV.

4.2.5 Third generation sequencing and future efforts

Two things characterize third the generation of sequencers: they are single molecule sequencers without any PCR steps involved and the reactions are observed in real time. Single-molecule real-time (SMRT) is the third-generation sequencing method developed by Pacific Bioscience. An active polymerase is immobilized at the bottom of each SMRT Cell, each patterned with 150,000 zero mode waveguide chambers, nucleotides diffuse into each of these chambers. In order to detect incorporation events and identify the base, each of the four nucleotides A, C, G and T are labeled with a different fluorescent dye having a distinct emission spectrum. Since the excitation illumination is directed to the bottom of the chamber, nucleotides held by the polymerase prior to incorporation emit an extended signal that identifies the base being incorporated. Read lengths of 20 KB and higher have been recorded and it is possible to generate base-modification data as well (Branton *et al.* 2008; Timp *et al.*, 2010).

The newest sequencer to come out is based on nanopore technology produced by Oxford Nanopore Technologies. They use a semiconductor chip with nanometer sized holes ('nanopores') to read DNA as it translocates through the pore under an electrophoretic current. The DNA is tethered to the pore by a polymerase which slows

translocation down to around 1000 bases-per-second (Ayub and Bayley 2012). The read lengths are expected to be 100,000 base pairs. In 2012, they announced the MinION sequencer, a disposable genome sequencer in a USB stick capable of generating over 1 GB of data (Niedringhaus *et al*, 2011).

The future of commercial sequencing will be driven by miniaturization of machines and lowering the costs as well as providing a high level of yield together with long reads. In addition, methods to handle to high level of data are being developed in parallel as well as novel techniques of processing and analyzing the data.

4.2.6 The bioinformatics requirements of handling next-generation sequencing data

With the development of many high-throughput technologies to be used in genome expression profiling, there was also a need for dedicated bioinformatics methods for these data. Some important steps are shared by all the different approaches and types of data, starting from obtaining the raw data, checking for quality and mapping to the reference genome. Most of the modern sequencers follow the same standard file formats. The reads obtained from the sequencers are presented in a FASTQ format, a text based format that presents the sequence and its sequencing quality (Cock *et al*, 2010). The range of quality values is different for each sequencer, but in principle quality score is an indication of probability of the base call being incorrect.

The FASTQ format is usually the input format for the next basic step, which is mapping or aligning the data to the reference genome. The reference genome is the current genome assembly released by the Genome Reference Consortium (<http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/>). Next-generation sequencing generally produces short reads, meaning short sequences of <~200 bases (as compared to long reads by Sanger sequencing, which cover ~1000 bases).

To compare the sequenced sample to its reference sequence, we need to find the corresponding part of that sequence for each read in our data.

Many different mapping tools are freely available and usually the choice of which to use depends on the exact dataset and technology used. In Paper I we used the Nexalign (Lassmann, T., <http://genome.gsc.riken.jp/osc/english/dataresource/>) while in Papers III and IV we used Delve, a probabilistic mapper. Delve uses a pair hidden Markov model to iteratively map reads to the genome and estimate position dependent error probabilities. After all error probabilities are estimated, individual reads are placed to a

single position on the genome where the alignment has the highest probability to be true according to the model. Phred scaled mapping qualities (Li *et al*, 2008), reflecting the likelihood of the alignment at a given genome position, are also reported, allowing filtering of the mapped reads based on the error probability of the mapping. Other popular mapping tools include BWA (Li and Durbin, 2009) and Bowtie (Langmead *et al*, 2009).

Possible errors during the mapping process can arise from the following: PCR artifacts from the early amplification steps of the sample, these errors are visible as mismatches in the alignment; sequencing errors come from the machine making an erroneous call either for physical reasons (limitations on the slide) or due to repeated stretches of the same base; mapping errors that arise due to the mapping algorithm, most often happening around repeat or low complexity regions (Li *et al*, 2012).

The mapping tools usually give output in the standard SAM/BAM file format. SAM format is the text version of the binary BAM format. These files include information about the alignment of the sequence and the mapping quality. They can easily be converted into the one another or queried for information using the samtools package (Li *et al*, 2009). Data files processed in this way can be further analyzed to answer specific questions as well as visualized and compared to existing data in dedicated genome browsers such as the UCSC Genome Browser (Meyer *et al*, 2013).

5 CONSORTIUM WORK ON TRANSCRIPTOME STUDIES

There is still a lot of work left to gain complete insight into transcriptional regulation and events in the cell. Similarly to the Human Genome Project, using the expertise and technology of different research centers, a few consortia aiming at demystifying transcriptional regulation have been formed.

5.1 THE FANTOM PROJECT

The Functional ANnotation of The Mammalian Genome project (FANTOM Consortium) begun in the year 2000 to assign functional annotations to the full-length cDNAs that were collected during the Mouse Encyclopedia Project at RIKEN, Japan. FANTOM has since developed and expanded over time to encompass the fields of transcriptome analysis involving researchers from 19 countries. The object of the project is moving steadily up the layers in the system of life, progressing from an understanding of the ‘elements’ - the transcripts - to an understanding of the ‘system’ - the transcriptional regulatory network. Since FANTOM3, the consortium has taken on CAGE as its main technology. Some of the major findings through the projects include: FANTOM3 revealed that 63% of the genome, instead of the previously thought 1.5% comprising of protein coding genes, is transcribed as RNA in the mammalian genome (mouse and human), as well as discovering over 23,000 non-coding RNAs and abundant sense-antisense transcription (Carninci *et al*, 2005; Katayama *et al*, 2005). FANTOM4 used CAGE adapted for next generation sequencing (Roche 454 machine) and the THP1 cell line to monitor the dynamics of TSS usage during a time course of monocytic differentiation. The expression levels from each promoter and TFBS predictions were then used to build a transcriptional regulatory network (Suzuki *et al*, 2009). The current project FANTOM5 aims to expand on previous projects to generate a map of the majority of human promoters and comparative transcriptional regulatory models across different primary cells, cell lines and tissues. The CAGE sequencing is performed on Helicos single molecule sequencer and RNA isolated from every major human organ, over 200 cancer cell lines, 200 primary cells as well as time courses.

The FANTOM resources have been used in several important research projects, including the Human Genome Project and the iPS cell establishment. The deliverable of FANTOM also include the FANTOM database and the FANTOM full-length cDNA clone bank.

5.2 THE ENCODE PROJECT

The ENCyclopedia Of DNA Elements (ENCODE) Project launched by the US National Human Genome Research Institute in September 2003 aims at identifying all functional elements in the human genome sequence. The pilot phase of the Project was focused on a specified 30 megabases (□1%) of the human genome sequence and is organized as an international consortium of computational and molecular laboratory-based scientists working to develop and apply high-throughput approaches for detecting all sequence elements that confer biological function. For the current phase, the primary assays used in ENCODE are ChIP-seq, DNase I Hypersensitivity, RNA-seq, CAGE and assays of DNA methylation. The combination of these technologies across cell lines enabled the project to assign biochemical functions for 80% of the genome, in particular outside of the well-studied protein-coding regions (Dunham *et al*, 2012; Djebali *et al*, 2012). Other deliverables include a comprehensive map of DNase I hypersensitive sites (Thurman *et al*, 2012), a lexicon of short DNA sequences that form recognition motifs for DNA-binding proteins (Neph *et al*, 2012), and a preliminary sketch of the architecture of the network of human transcription factors (Gerstein *et al*, 2012). One part of the ENCODE project is also GENCODE, a project to annotate all evidence-based gene features in the entire human genome at a high accuracy (Harrow *et al*, 2006).

6 APPLICATIONS TO DISEASES

An integral next step is to apply all of these technologies and approaches to research complex diseases. In my research I have applied these approaches to genes involved in dyslexia (Paper II) and Rett syndrome (Paper IV).

6.1 DYSLEXIA

Dyslexia is a common impairment in learning to read and write despite normal intelligence and normal senses that affects approximately 5%–10% of the population (Petryshen and Pauls, 2009). Developmental dyslexia is a complex neurodevelopmental disorder with a genetic basis. Many candidate genes for dyslexia have been identified with DYX1C1, dyslexia susceptibility 1 candidate 1, being one of the most promising. Function of DYX1C1 is mostly unknown, but it has been demonstrated to affect neuronal migration and modulate estrogen receptor signaling (Wang *et al*, 2006; Massinen *et al*, 2009). Knowledge that RNAi mediated knockdown in rats influenced cognitive processes (Threlked *et al*, 2007), is involved in estrogen receptor signaling and that the protein product can be seen in the nucleus, led us to think DYX1C1 would be a good candidate for perturbation studies.

6.2 RETT SYNDROME

Rett syndrome is a neurodevelopmental disorder that affects the grey matter of the brain. While almost exclusively affecting females, it has been detected in males as well. The clinical features include small hands and feet and a deceleration of the rate of head growth including repetitive stereotyped hand movements, seizures, no verbal skills or walking skills as well as intellectual disability (Neul *et al*, 2010).

It is caused by mutations in three functionally diverse genes: FOXP1 (Ariani *et al*, 2008), MECP2 (Amir *et al*, 1999) and CDKL5 (Weaving *et al*, 2004). Although the functions of FOXP1, MECP2 and CDKL5 have been studied individually, not much is known about their relation to each other with respect to expression levels and regulatory regions. Using the big data repository of FANTOM5, we set out to identify the promoter characteristics of each gene in human and mouse, as well as the other possible common features related to the core promoter region.

7 AIMS

Employing genome-wide profiling methods we aim to study in depth the promoter regions to characterize and better understand transcriptional regulatory events in the context of expression changes and using these approaches to study disease on the transcriptional level.

7.1 INDIVIDUAL AIMS

Paper I Combining knock-down and deepCAGE to infer genome-wide effects of the transcription factors.

Paper II Apply the method used in paper I to *DYX1C1* and infer the genome-wide effects of its perturbation.

Paper III Using human post-mortem brain samples, define promoter-level differences on the spatio-temporal level using single-molecule sequencer CAGE data.

Paper IV Using the wide set of samples, define the promoter regions, expression levels and shared regulatory level features of 3 genes implicated in Rett syndrome.

8 PRESENT INVESTIGATION

8.1 COMBINING SIRNA PERTURBATION AND DEEPCAGE GIVES US INSIGHT INTO TRANSCRIPTIONAL REGULATORY NETWORKS (PAPER I)

In this paper, we knocked down the 4 key transcription factors IRF8, MYB, PU.1 and SP1 in the human monoblastic leukemia cell line THP-1 (Tsuchiya *et al*, 1980). The capped RNAs in the knockdown and control samples were captured by CAGE and sequenced using the Roche 454 sequencer.

Since microarray experiments done on the same RNA samples were available through the FANTOM4 project (Suzuki *et al*, 2009), first we compared the fold changes of the perturbed genes for both the microarrays and CAGE. We found an overall positive correlation for all four TF knockdown samples across both technologies. In general, CAGE fold changes were greater than those measured by microarrays, as has been previously noted (deHoon and Hayashizaki, 2008).

Knockdown of SP1, IRF8, PU.1 and MYB led to induction of 267, 347, 189 and 307 genes and repression of 428, 527, 260 and 1160 genes by 1.5-fold up- or down-regulation, respectively. We used the top 50 of each set of perturbed promoters to search for novel motifs using de-novo motif finder MEME (Bailey *et al*, 2006). Our results were consistent with the expected roles of the TFs. For example, we find that knockdown of IRF8, a known activator (Meraro *et al*, 2002), results in down-regulation in both the deepCAGE and microarray experiments of XAF1, a gene which we predict to contain our novel motif. The observation that MYB knockdown yielded motifs for both up- and down-regulated sets is consistent with its known role as both a transcriptional activator and repressor (Luscher and Eisman, 1990).

We assessed if our motifs truly describe functional sites by comparing the expression fold changes of the TSSs containing the motifs. Since we had replica for microarray data only, we used microarray expression for these measurements, although we got no discernable differences when using just the CAGE values. We found the most interesting results in the IRF8 and PU.1 down-regulated sets and the motifs we found there. Promoters containing those motifs were expressed at significantly lower levels than promoters lacking the motif. Furthermore, our motif out-performed known motifs present in the Transfac database (Matys *et al*, 2006) for PU.1 and IRF8, as well as PU.1 ChIP data from the same cell line. Checking the conservation of our motifs, we find that 32.8 % (PU.1) and 35.5% (IRF8) of their base positions are strictly conserved

compared to the 3–8% average overall conservation and 11–24% conservation in coding regions.

Our found motifs were longer than the known ones present in the databases. Tested this by truncating the motifs to be of the same length, we lost specificity leading us to confirm our longer. Additionally, we found an overlap between down-regulated TSSs in both PU.1 and IRF8 (44 TSSs) that contain both motifs overlapping each other. Here, we found confirmation by already published data on the combinatorial regulation properties of these two TFs (Meracki and Fenton 2000; Meraro *et al*, 2002).

Finally, we constructed a promoter based gene regulatory network led by our confirmation that the most down-regulated genes contain the motifs linked to TFs. In this network, we included only genes that were perturbed upon knockdown of at least 2 out of 4 TFs. Genes co-regulated by PU.1 and IRF8 were predominantly co-downregulated upon knockdown. Interestingly, there is an antagonistic relationship for genes co-regulated by PU.1 and MYB, with the majority downregulated upon PU.1 KD but up-regulated upon MYB knockdown.

In this paper we have established the knockdown (KD) CAGE technology and demonstrated that it can be used to determine de novo TFBS as well as build promoter based networks. Compared to ChIP technology, we can identify sites that are influenced by TF KD, with ChIP identifying sites where the TF is bound but not necessarily functionally active (Wasserman and Sandelin, 2004). However, combining these two approaches could create a powerful method to discriminate indirect targets from direct targets bound by factors at both proximal and distal sites including enhancers and insulators.

8.2 PERTURBATION STUDIES OF DYX1C1 REVEAL ITS INVOLVEMENT IN NEURONAL MIGRATION PATHWAYS (PAPER II)

In the previous paper we explored the properties of knockdown of known TFs to infer regulatory networks of perturbed genes. In this study, we applied this knowledge to DYX1C1, a dyslexia candidate gene and analyzed molecular networks of DYX1C1 with global transcriptome and protein interaction assays.

We perturbed DYX1C1 in the neuroblastoma SH-SY5Y cells and investigated the global transcriptome changes using the Illumina HT-12v4 expression beadchip arrays. We detected 379 probes corresponding to 357 genes with significant differential expression in the DYX1C1 overexpressed cell line compared with control SH-SY5Y, 207 of these probes were up-regulated, and 172 were down-regulated. Genes previously

linked to neuronal differentiation and migration, RELN and ASL1 (D'Arcangelo *et al*, 1999; Dixit *et al*, 2011), were the most upregulated and downregulated. In the siRNA samples, siDYX1C1 was compared with siControl, revealing 88 differentially expressed probes corresponding to 87 genes, of which 15 probes were upregulated, and 73 were downregulated. In total, 30 genes including PDGFRA, SNAP91, CUX2, GAL, IL11RA, OLFM1, and PDS5A were differentially expressed in both comparisons. To check these genes for common features and put them into context, we examined the gene ontology (GO) enrichment. The upregulated genes of the overexpression experiment showed the most significant enrichment in the biological process GO terms “cellular component movement”, “cell migration”, and “nervous system development”. Interestingly, a total of 18 genes were classified in the “cell migration” term, and 6 of those genes (TWIST1, RELN, PHOX2B, NRCAM, DCX, and PXMP3I) are part of the “neuron migration” term, which is another confirmation of previous involvement of neuronal migration role of DYX1C1 (Wang *et al*, 2006). Additionally, the GO term “nervous system development” was also significantly enriched in the siDYX1C1 list of downregulated genes, with a total of 17 of the 73 genes in that term. The “cell migration” GO term was not significantly enriched when analyzing the siDYX1C1 downregulated gene list but, in the cellular component category, the “cell leading edge” term was significantly enriched, suggesting that also knockdown of DYX1C1 affects genes important for cell migration. We also looked for enrichment in the Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) and found overrepresentations for genes in “cell cycle pathway” for both experiments. Interestingly, the “focal adhesion pathway” was also upregulated significantly in the overexpression cell line. Given that focal adhesion constitutes a core machinery of cell migration (Lock *et al*, 2008), this further strengthens the notion that DYX1C1 controls cell migration. Another significant term present in both experiments was “pathways in cancer”, connecting to the previous suggestion of DYX1C1 being involved in breast and colon cancers (Chen *et al*, 2009; Kim *et al*, 2009).

To better characterize DYX1C1's function and involvement in pathways, we looked for its protein interacting partners using co-immunoprecipitation combined with protein identification with nano-liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). We identified 66 new proteins associated with DYX1C1, 24 of which were identified in at least two independent experiments. To infer the DYX1C1 protein interaction network, we searched Pathways commons database (Cerami *et al*, 2011) and found that 43 of the proteins in the DYX1C1 interactome have

previously been shown to interact with each other. The high connectivity of the proteins suggested that DYX1C1 might participate in multiprotein complexes. Again, we checked for GO term enrichment and found that “microtubule based process” and “microtubule organizing center organization” were among the most significant biological processes, again pointing toward a role for DYX1C1 in cell migration. The analysis of GO category “cellular component” showed a significant overrepresentation of cytoskeletal proteins, more specifically microtubule proteins among the DYX1C1 associated proteins. Centrosomal proteins such as CEP170, CENPJ, and NPM1 were among them and in other results we could also find that iDYX1C1 localizes to the centrosome when overexpressed. These characteristics are similar to those of other neuronal migration genes that promote the recruitment, stabilization, and organization of microtubules and actin that eventually drive neuronal migration and cell division (Liu SJ, 2011).

Additionally in this work, we identified a novel highly conserved protein domain in DYX1C1 of 43 residues that we called the “DYX1 domain”. Position specific iterated-blast searches did not detect this domain in any other protein family.

8.3 THE PROMOTER LEVEL ATLAS OF SPECIFIC TRANSCRIPTS AS WELL AS SPATIO-TEMPORAL COMPARISONS IN HUMAN BRAIN (PAPER III)

As part of the FANTOM5 project, a set of 15 brain regions from post-mortem individuals, one infant and 3 adults, were sequenced using CAGE on the Heliscope single molecule sequencer.

Using the advantage of all FANTOM5 samples profiled with the same technology, we compared the brain samples to all the other samples in the FANTOM5 human tissue collection. Using multi-dimensional scaling, we could see clear difference between brain samples and all the other samples. Looking at data subsets, such as only expression TF, enhancer expression, non-coding transcripts or expression coming from repeat regions, we were able to consistently observe this difference between brain and other tissues. These results show that the brain specific expression signature is distinctive with respect to other tissues, not only on the level of coding genes but also on non-coding transcripts or transcriptional regulators.

Attempting to explain these differences, we compared the cumulative distribution of tags accounted for by the 10,000 most highly expressed TSSs in each tissue sample. The obtained results suggest that brain has a more complex and diversified

transcriptome compared to other tissues, which is additionally proven with calculations of transcriptional complexity where brain samples significantly differ from and score higher than other tissues. Through differential expression analysis between brain and other tissues, we observe that one third of all transcripts are more highly expressed in brain and thus brain-specific. GO term analysis in this set reveals the terms for neurological system processes and transmission of nerve pulse. Additional important differences between brain and other tissues are brain specific alternative promoters. We identified 589 mRNA isoforms for genes expressed in brain but not in other tissues including some TFs.

Our samples comprised of one infant individual and 3 adults. To look for detailed differences on the temporal level, we performed again differential expression analysis between these two groups. Notably, over a quarter of the genes expressed in brain contain at least one differentially expressed TSS; most of these genes display both an infant or adult specific TSS as well as a non differentially expressed TSS (82.1%), while the remaining TSSs are exclusively expressed in one of the two age extremes. This shows that alternative TSS usage importantly contributes to the differences observed between infant and adult transcriptomes and suggests that the expression of specific mRNAs is differentially regulated in the two age extremes. Another interesting result is that about 20% of differentially expressed TSSs originate from non-coding RNA loci and 11% from intergenic regions, meaning that a lot of the difference comes from previously uncharacterized TSSs or those stemming from genes with unknown function.

In order to assess individual differences in expression between brain regions in the adult, we performed differential expression analysis between all the pairs of regions available. The brain region with the largest number of differentially expressed TCs and the largest fold changes is cerebellum. We observed that expression signatures were not characteristic of a single region but tended to be shared by groups of regions with related developmental derivation, functions or projections, consistent with previous reports (Hawrylycz *et al*, 2012). We separated the signal then in 4 distinct groups, each of them containing genes that are known markers for the regions they are expressed in: 1) frontal, temporal, parietal and occipital lobes, hippocampus and amygdala (cortex- limbic system) 2) caudate and putamen (striatum), 3) cerebellum, 4) thalamus, globus pallidus, substantia nigra, locus coeruleus, spinal cord and medulla oblongata (brain stem-basal ganglia). For each of these groups we identified TFs that are differentially expressed. Often these TFs were enriched in more than one group but these differences

were driven by group specific alternative promoters. We also built regulatory interaction networks for each TF group and were able to confirm known interactions for the majority of TFs in the group (average of 66%).

Overall we have classified brain specific expression signal on the promoter level into different sections (adult, infant, brain region group), which leaves us with 67% of genes that are highly expressed in brain when compared to other tissues as the brain expression signal not explained by any other comparison. We have also pointed out that many of the differences in brain expression result from poorly annotated or poorly characterized regions of the genome. Further research in this regions could give novel findings of brain specific genes.

8.4 CHARACTERIZING RETT DISEASE GENES ON THE PROMOTER LEVEL (PAPER IV)

In contrast to the previous study that was based on characterizing various aspects of and contrasting a vast amount of, data, in this study we picked 3 genes specific for Rett syndrome. We used the FANTOM5 data for human and mouse for in detail characterization the promoters, regulators and expression patterns of these three genes.

First, we identified CAGE-derived TSSs for all 3 genes in both human and mouse. We identified a novel main promoter in mouse for Foxg1. Both human and mouse had high expression of Foxg1 in brain, but intriguingly, expression was absent in the cerebellum samples. Analyzing ENCODE DNase-I hypersensitive sites and active promoter histone marks (H3K4me3) available for mouse, we found evidence of silencing of FoxG1 by the PRC2 complex in the cerebellum. For Mecp2, we identified two TSSs in both human and mouse (with an exception of a third lowly expressed TSS expressed exclusively in blood primary cells, particularly in CD14 monocytes). We found Mecp2 to be ubiquitously expressed in all tissues and not just the brain. For Cdk15 we also found 2 TSSs for both species, also expressed ubiquitously.

We compared all the TSSs of these 3 genes for correlations in expression levels and found that Mecp2 and Cdk15 are more highly correlated to each other than they are to Foxg1. Additionally, the expression of main TSSs of Foxg1 and Mecp2 are in contrast to each .

Using Encode ChIP data we additionally characterized the mouse promoters for the presence of enhancer or promoter markers. For all the main promoters of the 3 genes, we find enhancer marks close by in the mouse, while for human, using the enhancer

data of FANTOM5, we find 4, 14 and 1 significantly correlated enhancers for FOXP1, MECP2 and CDKL5 respectively.

When we characterized the promoter shapes of all the TSSs, we found that most of them are broad, consistent with their connection to CpG islands. We also found a strong conservation of promoter shape across species, particularly visible in the highest expressed TSS of FOXP1.

We calculated also the probabilities of having the same TFs regulate the three genes. Our data reveal that the sequence region around the main promoter of FOXP1 in human is significantly enriched in binding sites for the RREB1 ($p = 0.01$), FOXP1 ($p = 0.03$), and NFY ($p = 0.01$) transcription factors. NFY is also predicted to regulate MECP2 ($p = 0.01$) and possibly CDKL5 ($p = 0.09$). In mouse, for all three genes the promoter regions are enriched for motifs associated with transcription factor NFY, as well as Sp1.

FOXP1 and MECP2 both are TFs and thus binding DNA at the promoter regions. Using the expression data of the whole FANTOM5 we checked their motif activities (ref). Although FOXP1 is expressed mostly in brain tissues, its motif activity did not show any significant difference between brain and other tissues in either human or mouse (human $p \leq 0.2738$, mouse $p \leq 0.3272$). In contrast, the motif activity of MECP2 is significantly lower in brain compared to other tissues both in human and mouse (human $p \leq 1.019e-10$, mouse $p \leq 0.0005343$), consistent with a role of MECP2 as a negative regulator in brain.

Our comprehensive analyses of the data from the FANTOM5 project reveal that although the three genes are related by disease phenotype, their genomic architecture, expression and regulation are independent of each other. Thus intersecting molecular pathways likely cause the overlapping phenotypes seen in Rett patients.

9 CONCLUSIONS AND PERSPECTIVES

Through these papers we used the power of CAGE in conjunction with next-generation sequencing to define the promoter region of genes, regulatory element binding sites as well as alternative promoter usage depending on different states.

In paper I we developed a method combining siRNA knockdown and sequencing based CAGE to show we can accurately predict genomic loci where TFs are actively regulating transcription. We then applied this same approach to a gene linked to a disease but without completely known function and without a known DNA binding site. Even without a TF function, we could still derive the downstream effects of perturbing *DYX1C1*.

For papers III and IV we used the FANTOM5 database of tissues and cells. In paper III we used this to make global conclusions about differences in regulation between i) brain and other tissues, ii) adult and infant brain as well as iii) different adult brain regions. Using an opposite approach, in paper IV we started with 3 previously not associated genes and investigated their detailed promoter-level functionality using the database of tissues and cells. This way we have shown how the same data repository can be used for more global questions as well as specific questions such as the common characteristics of three genes.

This approach has shown us that genome-wide studies and quantitative measures can give a glance at to why the cells are different and what might be the underlying mechanisms. To gain further details, additional studies that would involve complementary methods such as RNA-Seq to give us an idea if the alternative TSS we find do give different protein products in the end. Of course, in complex disorders we should not forget the potential influences of other factors including non-coding RNAs, enhancers, epigenetics or repeat regions.

The advent of next-generation sequencing has brought us into an era of vast biological data. The advances in sequencing technologies and lowering of costs in general has brought us closer to clinical application of these technologies. Main goal in this area is to develop diagnostic tools that medical doctors can use in everyday work with patients to quickly diagnose or classify a disease. This would particularly be necessary to end

the 'diagnostic odyssey': the grueling, painful, expensive and sometimes decades-long journey from negative test to negative test, failing to diagnose a rare disease. Currently methods are being developed with exome sequencing as a diagnostic tool with 25% of diagnosis rate for now (Glusman G, 2013). Additional diagnostic methods can rely in developing un-invasive ways to diagnose a patient that would normally require invasive procedures, such as identifying markers in the blood that are quick to be checked by clinical personnel. The miniaturization of sequencing machines is also leading to this point that desktop sequencers for small samples would be available to clinicians.

Good communication needs to be established between the lab and the clinic for both to profit from it. The clinician could this way provide valuable disease samples which in turn could lead to better diagnostic methods or even potentially to identify intervention points for drug treatment.

Another novel topic in this field, which arose due to lowering costs of sequencing and the ability to take small amounts of RNA, is single cell sequencing. It is a common perception that expression in a tissue is an average of the expression of all the single cells making it up. First single cell studies have found genes can be highly expressed in some cells while they are virtually not at all expressed in other cells of the same cell type possibly resulting from expression bursts. Not only is the biology of single cells still poorly understood but also the methods required to obtain single cells (often manually) are complicated and it is hard to separate cells but still keep the information about their cross-talk to be visible in the sequencing step.

The methods described here are basic science that is gradually turning toward clinical samples and clinical application. Hopefully, the average patient will be able to soon benefit from these services and have the diagnostics and treatment be speeded up with these technologies.

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