# Chapter X: Groucho activity in the developing embryo

## Abstract

## Introduction

Groucho is a crucial regulator of gene expression throughout development and is centrally involved in the establishment of embryonic patterning in the early *Drosophila* embryo. Through its interaction with a number of sequence-specific transcription factors, Groucho is crucial to the spatial and temporal restriction of gene expression beginning very early in development, and remains involved in tissue differentiation and specification throughout larval and pupal stages. As Groucho is maternally deposited, high levels of the transcript are present from the onset of development, allowing Groucho to play a role as one of the earliest translated proteins in the embryo. Groucho is one of a group of factors that enables a cell to alter its transcriptional profile, and therefore developmental fate, in response to informational signals arising outside of the cell. This regulatory role of Groucho homologues is conserved throughout metazoans. And, due to Groucho’s highly-connected position in the developmental regulatory network, mutations in the function or regulation of Groucho can result in developmental abnormalities and disease.

Despite the extensively documented centrality of Groucho to multiple developmental processes, especially in early embryonic development, no systematic genome wide investigation has been undertaken to precisely position Groucho into the developmental regulatory network. To this end, we have adopted a multi-omics approach to identify Groucho target genes with high-confidence and at discrete times in early Drosophila development. The timepoints used for the analysis were chosen to overlap significant events in embryonic development that have known Groucho interactions.

The first timepoint (1.5 – 4 hours post deposition) encompasses formation of the syncytial blastoderm and subsequent cellularization. It is during this stage that the expression patterns of the pair-rule and segment polarity genes (including engrailed, a Groucho interacting TF) are established. The second timepoint (4 – 6.5 hours) encompasses the growth and segmentation of the germ band, including the formation of neuroblasts. Timepoint 3 (6.5 – 9 hours) encompasses retraction of the germ band and fusion of the anterior and posterior midgut.

## Materials & Methods

## Results

*Groucho is recruited ubiquitously and dynamically throughout the genome*

ChIP-seq was performed in duplicate on fly embryos collected at three timepoints using an affinity-purified polyclonal Groucho antibody. Sequencing libraries were sequenced to a depth that provided at minimum 5M uniquely mappable reads, far in excess of the minimum recommended by modENCODE ChIP-seq best-practices (Fig. 2-a//A) {Landt, 2012 #308}. Replicates exhibited high reproducibility in terms of both read density and resulting peak models. (Fig. 2-a//B, left and right, respectively). Peak modeling identified widespread Groucho binding throughout the genome; peaks with overlapping regions between replicates were chosen for further analysis (Fig. 2-b). Groucho recruitment sites appear most numerous during the central timepoint analyzed (5,246 non-overlapping binding sites), compared to the early (1,358) and late (4,232) stages. We detected 5,829 unique binding sites in total, with 535 sites recruiting Groucho at all timepoints, and therefore potentially participating in Grouch-mediated repression in at least one cell type or tissue throughout the developmental timeframe analyzed (Fig 2-c).

Groucho occupancy is highly dynamic and reversible. Approximately 50% of all Groucho binding sites are unique to a single timepoint. The majority of the sites established during timepoint 1 that persist into timepoint 2 continue to persist into timepoint 3, indicating that some Groucho binding sites are utilized throughout early development. Interestingly, no sites are occupied in only the first and third timepoints, indicating that Groucho occupied sites during the first timepoint tend to either be utilized at all timepoints, or are only utilized very early in development and not utilized again.

*Groucho-associated genes tend to be associated with multiple, small clustered peaks*

Groucho binding regions are associated with a smaller number of genes (Fig. 2-d//A), due to the tendency of Groucho to localize to multiple regions around its potential targets. Half of all Grouch-associated genes have two or more Groucho peaks in relative proximity (Fig. 2-d//B). These peaks have median widths in the 500 – 700 bp range, indicative of point source peak, as commonly seen for sequence-specific transcription factors, rather than the broad peaks typical of polymeric factors or histone marks. However, at all three timepoints, the distribution of peak widths exhibits a prominent tail of much wider peaks in the 1.5 to 2.5 kb range. This indicates that, consistent with previously proposed models of Groucho binding, it may be capable of spreading over relatively large regions of the genome, but this does not appear to be a common mode of chromatin association. Average Grouch peak widths slightly increase at later timepoints, though whether this is indicative of a time-dependent change in the way Groucho interacts with chromatin, or slight differences in library composition, is unclear.

Groucho binding is enriched in promoter regions within 500bp of transcription start sites (Fig. 2-e//A). Regions further upstream (20 kb to 2 kb) are depleted for Gro occupancy, and intermediate range regions (2 kb to 500 bp) show neither depletion or enrichment. Binding within introns and 5’ UTRs is enriched. This pattern of occupancy is at odds with the traditional view that Groucho is primarily a long-range corepressor {Dubnicoff, 1997 #2366} {Barolo, 1997 #2365}, capable of repressing genes several kilobases away from its recruitment site, though it has also been shown to be capable of short-range repression through recruitment by additional transcription factors, such as knirps {Payankaulam, 2009 #2955}. Our data indicates that it preferentially associates near TSSs and within genes. Groucho sites exhibit a strong preference for binding within genes, with approximately 50% of peaks occurring within gene bodies across all timepoints (Fig. 2-e//B). A small fraction of binding overlaps start sites (3 – 10%), and intergenic binding is nearly evenly split between binding upstream and downstream of the nearest feature. Due to the complexity of enhancer-gene interactions, it is difficult to assign these intergenic binding sites accurately to a regulatory target, if such a target even exists for each peak.

Within gene bodies, Groucho exhibits a strong preference for binding within introns and UTRs, and is depleted for exon binding when compared to input (Fig. 2-g//A). Between 60 and 80% of all binding within genes occurs within introns, dependent on timepoint. Of all Groucho intornic binding sites, 40% fall within the first intron. This represents a more than 2-fold enrichment of binding preference for these introns, and is consisten with the observation that the first introns of *Drosophila* genes tend to be longer, more conserved, and more sensitive to mutation than subsequent introns {Bradnam, 2008 #3034}.

*Groucho spreading does not appear to be an obligate feature of repression*

. More recently, ChIP-seq data obtained for Groucho binding in S2 and Kc167 cells showed that a significant fraction of Groucho binding sites overlap transcription start sites (25% and 40% of sites, respectively). ChIP-seq in early embryos exhibits a shifted distribution of Groucho binding (*Fig. bar chart of binding site occupancy relative to genes and density graph around TSS*), with at most 8% of Groucho binding sites overlapping TSS regions. Our data suggests Groucho exhibits higher fractional occupancy of intergenic regions in embryos, with a smaller percentage of Groucho binding inside gene bodies than in the two cell lines studied. This differenc in site occupancy could be explained by the different developmental contexts of the embryos and cell lines studied. The two cell lines studied are derived from later embryonic stages (stages 16-17 for S2, and stages 13-15 for Kc167). Our ChIP-seq data represents an amalgamation of numerous cell types, each potentially expressing a subset of Groucho-recruting factors. This may explain the discrepancy in TSS binding, as more numerous and diverse repressor presence in the early embryo compared to single cell types would result in more distributed Groucho binding.

*Identification of Groucho Targets by Developmental Stage*

Groucho is a crucial regulator of gene expression throughout development and is centrally involved in the establishment of embryonic patterning in the early embryo. While several Groucho regulatory targets are known, it is suspected that the majority of Groucho targets have yet to be identified. To this end, we analyzed the transcriptomes of staged embryos expressing multiple dosages of Groucho. These included fly lines maternally overexpressing Groucho at two levels, two-fold and four-fold higher than endogenous, as well as a line overexpressing a Groucho deletion mutant lacking the central SP domain (Gro∆SP). Additionally, we analyzed the transcriptome of embryos lacking maternally-contributed functional Groucho. These embryos express a severely truncated and non-functional form of Groucho.

Perturbation of Groucho levels results in the misregulation of a significant proportion of the Drosophila genome over each timespan (Fig 2-k). The Groucho loss-of-function phenotype was more severe than that obtained from overexpression, with over 10% of genes exhibiting changes in expression level at each timepoint. As Groucho is known to restrict the expression patterns of several transcription factors (including tailless and huckebein), it is suspected that many of these potential Groucho targets are secondary targets of Groucho and are not regulated by direct Groucho occupancy of their enhancer regions.

Though the Groucho/TLE family of proteins have traditionally been thought of as obligate repressors, TLE3, a human Groucho ortholog, was recently shown to primarily serve as an activator, though the mechanism remains unknown{Villanueva:2011ff}. Additionally, CtBP, a canonical, short-range *Drosophila* corepressor, was shown to serve as a co-activator of certain Wnt-regulated genes, this switch in behavior being controlled by the protein’s oligomeric state{Bhambhani:2011je}.

To identify primary targets of Groucho at each timepoint, we compared differential gene expression of every expressed gene under conditions of Groucho overexpression versus Groucho null. Genes which show an opposite magnitude change in expression under the two conditions were then considered for further analysis. At early timepoints, a greater percentage of genes appear to be repressed by Groucho than activated, with this trend reversing with increasing age. (*Fig : heatmap of selected gene expression changes in Gro MB36 embryos)* The significantly enriched gene ontology groups for predicted Groucho-repressed genes (n = 162) contain several groups indicative of transcription regulation (GO:0006355, n = 37) and developmental processes (GO:0032502, n = 81). (*Fig: GO groups)* Of the 146 predicted Groucho-activated genes, no gene ontologies were significantly enriched, leading us to hypothesize that these genes are potentially the result of random noise in the gene expression data and do not represent direct Groucho targets.

*Total mRNA levels correlate well with nascent mRNA levels at all timepoints*

As embryos at the stages utilized for transcriptome measurements are highly dynamic systems, with rapidly fluctuating levels of transcripts, we used Nascent-seq to confirm that the transcriptome measurements were indicative of actual transcription rates, and not overwhelmed by the various contributions of maternal mRNA contribution or differential rates of mRNA maturation and degradation. Sequencing of nascent RNA has been utilized to monitor fluctuating mRNA levels, for example following induction of an immune response in cell culture{Bhatt, 2012 #2995}. In *Drosophila*, nascent-seq has been used to monitor cotranscriptional splicing in adult flies{Khodor, 2011 #2081}, as well as circadian transcript cycling{Rodriguez, 2013 #1782}, in which the authors saw significant differences in total mRNA and nascent mRNA levels over ninety minute collections.

Embryos were collected at each timepoint and fractionated to isolate chromatin-associated RNA, which is enriched for nascent transcripts. Efficient fractionation was confirmed by immunobloting for cytoplasmic and nuclear components (*Supplemental figure*).

*Groucho-regulated genes are enriched for stalled RNA polymerase*

Promotor-proximal pausing of RNA Polymerase II has been identified as a crucial step in gene regulation. Pausing has been primarily characterized in *Drosophila* at multiple heat-shock genes, presumably to facilitate rapid induction of gene expression upon receipt of an appropriate regulatory signal{Lis:1993uk}. Since this discovery, polymerase stalling has been found to be a ubiquitous regulatory mechanism{Conaway:2000un}, with strong peaks of PolII present in the promoter regions of a diverse array of genes throughout the *Droosphila* genome.

To explore whether Groucho regulation potentially promotes the stalling of polymerase, we undertook to compare Groucho-regulated genes with publically available genome-wide PolII localization data. In this data set, the authors classified each gene into one of several states including the lack of detected PolII, active (elongation phase) PolII, or stalled PolII. Comparing genes exhibiting change in expression levels under Groucho loss-of-function conditions, we see a strong correlation between genes repressed by Groucho and PolII pausing ( 179 genes, *p* < 10-20), and limited correlation between genes activated by Groucho exhibiting pausing (68 genes, *p* > 0.05). Conversely, genes activated by Groucho are enriched for active PolII (315 genes, *p <* 10-20), while Gro repressed genes are not (174 genes, *p* > 0.01). Together, this provides strong evidence that, at least at early timepoints, a significant fraction of Groucho-associated genes exhibit characteristics of PolII pausing. The retention or prevention of PolII from transitioning to an active complex is a potential mechanism of Groucho-dependent repression.

## Discussion