*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: Table or heatmap of significant differential expression of each gene by timepoint*). 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:2012cc}. In *Drosophila*, nascent-seq has been used to monitor cotranscriptional splicing in adult flies{Khodor:2011hp}, as well as circadian transcript cycling{Rodriguez:2013kq}, 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 occupancy undergoes extensive rearrangement during early timepoints*

ChIP-seq was performed in duplicate on fly embryos collected at three timepoints with a polyclonal Groucho antibody. Peak modeling identified widespread Groucho binding throughout the genome, and high biological reproducibility between replicates (Figure – Venn diagram showing peak overlap between replicates per timepoint, as well as overlap of peaks between timepoints). Groucho occupancy appeared highest at the middle timepoint (5246 non-overlapping binding sites) compared to the early (1358) and late (4232) stages. These represent 5829 unique binding sites, with 535 sites constitutively occupied.

*Groucho recruitment sites exhibit large variability in width and distance from transcription start sites*

Groucho has traditionally been considered a long-range corepressor, capable of repressing genes several kilobases away from its recruitment site{Dubnicoff:1997we, Barolo:1997bh}, though it has also been shown to be capable of short-range repression through recruitment by additional transcription factors, such as knirps{Payankaulam:2009kn}. 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 differency 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.

*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.

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