# **Chapter 2: Groucho activity in the developing embryo**

## Abstract

Animal developmental patterning is a vastly complex and intricate process, requiring the integration of multiple temporally and spatially variant signals to define the transcription profile of each cell. The Groucho family of transcriptional corepressors play a crucial role in this process throughout the animal kingdom. In *Drosophila,* Groucho exerts control over gene expression via recruitment to the genome through the action of multiple DNA binding factors to which Groucho binds with tunable affinity. The recruiting factors are generally expressed and/or activated in spatially and temporally regulated domains , and therefore Groucho mediated expression is dependent on the presence and concentration of these factors. Groucho itself is ubiquitously expressed although its activity is regulated post-translationally at least in part via extracellular signals.

Despite the broad importance of Groucho in fly development, a full picture of its regulatory network in the developing embryo has yet to be established. To this end, we have undertaken a multiomics approach to identify Groucho targets during three discrete stages of embryonic development. At each stage, we have analyzed the embryonic transcriptome of wild-type and Groucho mutant embryos. Additionally, we have utilized high-throughput sequencing of chromatin-associated RNAs (nascent-seq) to confirm transcriptional rates at each timepoint and to determine if Groucho-regulated genes possess unique nascent-seq profiles. Groucho ChIP-seq provides information about the dynamics of the localization of Groucho to the chromatin in wild-type embryos. By combining these data sources, we gain mechanistic insights into how, when, and why Groucho is recruited to the genome. Additionally, we establish a temporally discrete high-confidence set of Groucho regulated genes, illuminating Groucho's multiple roles in developmental processes.

Groucho appears to be involved in the regulation of hundreds of genes throughout embryonic development, a significant proportion of which are regulatory genes themselves, reinforcing the idea that Groucho is a highly-connected node or hub in the developmental regulatory network. Groucho's recruitment is highly dynamic, with a widespread transition in genomic localization occurring after activation of the zygotic genome. While human homologs of Groucho have been shown to act as both coactivators and corepressors, Drosophila Gro appears to be a dedicated repressor. While Groucho has long been considered to be a long-range corepressor, often binding thousands of base pairs away from its target genes, we find that this is not an obligate condition for repression, as actively repressing Groucho is often bound directly adjacent to transcription start sites. Furthermore, while Groucho is known to oligomerize *in vivo*, the role and necessity of this oligomerization in repression remains unknown. We find that, while Groucho can bind over large portions of the genome, potentially through self-association, most binding events are more spatially constricted. While few long homogeneous stretches of Groucho are observed, Groucho-regulated genes do tend to exhibit clusters of multiple Gro associated peaks. Around some classes of regulated genes, Groucho exhibits binding to both distal and proximal regions, perhaps indicative of Groucho serving a role in facilitating contact between regulatory regions of the genome.

## Introduction

The corepressor Groucho (Gro) 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 numerous sequence-specific transcription factors (repressors), Gro is crucial to the spatial and temporal restriction of gene expression beginning very early in embryonic development and continuing throughout larval and pupal development. As Gro mRNA is maternally deposited in the oocyte, high levels of the protein are present from the onset of development, and as such Gro participates in many of the earliest transcriptional decisions in the embryo. Due to the highly-connected position of Gro in the developmental regulatory network, changes in Gro levels or function result in profound developmental abnormalities and disease.

In this study, we utilize high-throughput sequencing technologies to characterize the dynamics of Groucho genomic binding and to identify Groucho repressive targets. Global analysis of Groucho binding patterns via ChIP-seq allows us to gain insight into the mechanisms of Groucho-mediated repression via characterization of Groucho localization within and without genes, and how this localization correlates with binding patterns of additional transcription factors, including those known to interact with Gro. Analysis of the embryonic transcriptome under conditions of perturbed Groucho dosage enables us to dissect Groucho’s role in multiple signaling pathways and, integrated with the ChIP-seq analysis, to identify these targets and Gro participatory roles with high confidence.

Although Gro is ubiquitously expressed, it is not simply a passive component of the transcriptional machinery. For example, its function can be actively regulated in response to informational signals arising outside of the cell, through, for example, the action of the Ras/MAPK pathway. In addition, although Gro does not bind to DNA directly, it may participate via unknown mechanisms in target gene selection. This is demonstrated by a Gro deletion analysis in which it was shown that deletion of a Gro domain termed the SP domain results in promiscuous repression of genes not normally targeted by Gro.

Despite the extensively documented centrality of Gro in multiple developmental processes, especially in the early embryo, no systematic genome-wide investigation has been undertaken to position Gro in the fly developmental regulatory network. A more thorough understanding of the recruitment patterns of Gro in the early embryo, and the dynamics of such binding, will allow us to address multiple questions about the mechanism of Gro-mediated repression and the position of Gro in the regulatory hierarchy of pattern formation.

Gro tetramerizes and perhaps forms higher order oligomers *in vitro.* This together with the observations that Gro is required for long-range repression and that it binds core histones has led to the suggestion that Gro-mediated repression may involve spreading along chromatin. Indeed, in some contexts Gro oligomerization is necessary for repression *in vitro* {Chen, 1998 #267} and *in vivo* {Song, 2004 #1161}. However, it does not appear to be a universal requirement for repressive activity in all developmental contexts {Jennings, 2007 #2990}. Evidence from ChIP-PCR experiments suggests Gro spreads over potentially long stretches of chromatin presumably through its ability to self-associate {Winkler, 2010 #2964} {Martinez, 2008 #2287}, though these studies are limited by the resolution of the ChIP-PCR analysis. More recent Gro ChIP-seq data obtained from two Drosophila cell lines (S2 and Kc167) {Kaul, 2014 #2204} indicate that binding is primarily localized to discrete peaks in those cell lines. However, it is unclear to what degree that binding pattern extends to embryos. Genome-wide analysis of binding patterns in embryos presented in this thesis has enabled me to thoroughly investigate the requirement for spreading in Gro-mediated repression. We find that, while spreading, while Groucho is capable of spreading over long regions of chromatin, this spreading appears to be a rare feature of repression, with the majority of Groucho binding occurring in discreet peaks characteristic of recruitment to association with site-specific transcription factors. However, these discrete peaks often cluster over longer stretches of chromatin, indicative of the formation of loop-like structures.

The accurate assignment of a binding region detected by ChIP-seq to a specific regulatory target (or targets) is a long standing problem in the useful interpretation of ChIP-seq studies {Sikora-Wohlfeld, 2013 #2377}; the inaccuracy of association becomes more significant the further a factor binds from its regulatory target, as genomic complexity often makes assignment of enhancer-gene interactions uncertain. A common methodology to address this challenge is to incorporate genome-wide binding data with transcriptome measurements in systems perturbed for said factor {Dolinski, 2015 #3045}. To this end, we have employed RNA-seq to examine the effect of Gro-knockdown and Gro-overexpression on the transcriptome measurements at timepoints matching those used in the ChIP-seq analysis. When combined with the ChIP-seq binding profile data, this has allowed me produce a high-confidence set of Gro target genes at each timepoint, thus enabling me to thoroughly characterize the role of Gro during early development through a broad investigation of its influence on the developmentally-regulated gene network. The analysis to be presented here shows that Groucho targets are enriched for transcription factors, confirming its role as a pioneering factor in the establishment of cellular fate.

## Materials & Methods

1. *Groucho chromatin immunoprecipation (ChIP) and sequencing*
2. *Groucho ChIP-seq data analysis*

Multiplexed libraries were sequenced on Illumina HiSeq 2000 sequencing platforms (High Throughput Sequencing Facility, Broad Stem Cell Research Center, UCLA). Reads were demultiplexed via custom scripts. Demultiplexed libraries were filtered for read quality and PCR duplicates. Alignment was performed against the Drosophila melanogaster genome (iGenomes BDGP 5.25 assembly) with Bowtie2 (v2.2.5) using the following parameters: *-very-sensitive-local* {Langmead, 2012 #3049}. Peak calling was performed using MACS2 (v2.1.0) with default parameters {Zhang, 2008 #2203}. Peak visualizations generated with Integrated Genome Browser (v8.4.2) {Nicol, 2009 #3050}.

1. *Embryonic RNA isolation and sequencing (RNA-seq)*

Staged embryos were manually homogenized in TRIzol reagent (Life Technologies) according to manufacturer protocols. Purified RNA quality was assessed via Bioanalyzer 2100 (Agilent Technologies). polyA-selected libraries were generated with TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced on the Illumina HiSeq 2000 platform.

1. *RNA-seq data analysis*

Reads were demultiplexed via custom scripts. Low quality reads were trimmed and remaining reads were aligned with TopHat2 (v2.0.9) {Kim, 2013 #1817} against the *Drosophila melanogaster* genome (iGenomes BDGP 5.25 assembly) with iGenomes gene models as a guide. Gene assignment was performed with HTSeq {IAnders, 2015 #3027}. Differential expression analysis was performed with DESeq2 (v1.8.0) {Love, 2014 #3031}.

## Results

*Groucho is recruited ubiquitously and dynamically throughout the genome*

The time windows used for the analysis were chosen to overlap significant events in embryonic development that have known Groucho interactions. The first window (timepoint 1: 1.5 – 4 hours post-fertilization) 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, a defining step in anterior-posterior patterning. Specification of presumptive germ layers along the dorsal-ventral axis occurs during this stage, primarily guided by the activity of Dorsal in conjunction with Groucho. The second window (timepoint 2: 4 – 6.5 hours post-fertilization) encompasses the growth and segmentation of the germ band, including the formation of neuroblasts, a crucial early step in the onset of neurogenesis. The third window (timepoint 3: 6.5 – 9 hours post-fertilization) encompasses retraction of the germ band and fusion of the anterior and posterior midgut.

ChIP-seq was performed in duplicate on fly embryos representing each time point. We used an affinity purified polyclonal antibody raised against the Gro GP domain, which we validated extensively in immunoprecipitation and immunoblot assays. Sequencing libraries were sequenced to a depth that provided at minimum 5 million uniquely mappable reads, far in excess of the minimum recommended by modENCODE ChIP-seq best-practices (Fig. 2-1A) {Landt, 2012 #308}. Replicates exhibited high reproducibility in terms of both read density and resulting peak model (Fig. 2-1B, left and right, respectively).

The high degree of correlation between our ChIP-seq data sets and ChIP-chip data sets obtained from 0-12 hour embryos {Negre, 2011 #3035} using completely independent antibodies also validates our ChIP-seq data (Fig. 2-2). The modENCODE Groucho peaks were generated from 0 – 12 hour embryos and so should represent a time-averaged superset of our data. Collectively the ChIP-seq peaks from our three data sets, include xx% of the modEncode ChIP-chip peaks. Comparison of our ChIP-seq data with modENCODE Groucho ChIP-chip data generated from white pre-pupae also shows a significant overlap (Fig. 2-3). However, a large fraction of embryonic and pre-pupal binding sites are distinct from one another, consistent with the distinct roles of Groucho-mediated repression during early and late development.

Peak modeling identified widespread Groucho binding throughout the genome; peaks with overlapping regions between replicates were chosen for further analysis, as they represent a higher confidence subset of all identified peaks (Fig. 2-4). Peaks overlapping input peaks were removed, as they are assumed to arise from erroneous read alignment. 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 across all timepoints, and therefore potentially participating in Grouch-mediated repression in at least one cell type or tissue throughout the developmental timeframe analyzed (Fig 2-5).

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, few 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 in the stages analyzed.

*Groucho associates with multiple ventrally-repressed genes in patterns unique to early developmental stages*

In the early embryo, delineation of the dorsal-ventral axis is accomplished through transcriptional changes arising from a maternally-defined gradient of nuclear Dorsal (dl) along this axis {Roth, 1989 #1112}. In ventral and ventrolateral regions of the embryo, Dorsal facilitates the repression of numerous genes, including Zen (*zen*), Decapentaplegic (*dpp*) and Tolloid (*tld*) through its interaction with Groucho, a critical step in delineating presumptive mesodermal and neuroectodermal regions {Dubnicoff, 1997 #2366} {Kirov, 1994 #3107}. Ventral repression of *zen* is established through Dorsal recruitment to a so-called ventral repression region (VRR) between 1.1 to 1.4 kb upstream of the transcription start site. This region contains four Dorsal binding sites, as well as AT-rich regions responsible for the recruitment of Cut (*ct*) and Dead ringer (*dri,* also known as Retained, *retn*) {Valentine, 1998 #3036}. Through the cooperative action of these factors, Groucho is recruited to establish repression. ChIP-seq data confirms that Gro localizes around the VRR, however Gro density is comparatively weak within the VRR region (Fig. 2-6A). Stronger Gro signal is seen both directly upstream of the VRR (compared to *zen*), as well as downstream. This downstream region overlaps the TSS of *zen* and extends an additional 700 bp upstream of the gene. Following the 1.5 – 4 hour timepoint, Groucho recruitment to the *zen* locus shifts to a pair of peaks centered approximately 500 bp downstream of *zen*, and all upstream binding is lost. As no *zen* regulatory regions have been characterized 3’ of the gene, it is not known if these regions represent actively repressive Groucho. Though the mechanism has not been extensively documented, *zen* expression contracts in stage 5 (2 – 3 hr) embryos, and becomes permanently repressed afterwards. Overall, Gro binding at the *zen* locus confirms that Gro is recruited specifically to the VRR, as previously described, but does not remain confined to this position. This Gro-associated region then appears to associate directly with the TSS of zen, as well as several hundred bases upstream. It is unlikely Gro is directly recruited adjacent to the TSS, as this region is not sufficient for Gro-mediated repression, potentially indicating that Groucho occupancy at the TSS is initiated through spreading or looping from the recruitment site/VRR over adjacent regions, and that this recruitment.

Additionally, Dorsal is responsible for ventral repression of decapentaplegic (dpp) in early embryos (1.5 – 2 hours post fertilization) through the recruitment of Gro. Loss of Gro activity at this stage results in complete derepression of *dpp* in ventral regions of the embryo {Dubnicoff, 1997 #2366}. Dorsal binding sites necessary for restriction of *dpp* expression to the dorsal portion of the embryo have been mapped to the second intron {Huang, 1993 #3037}. Our ChIP-seq data confirms extensive Gro recruitment to this site (Fig. 2-6B), indicating also that Groucho is lost from this locus at later timepoints, consistent with the finding that in later embryonic development multiple elements upstream of *dpp* confine its expression into multiple lateral stripes along the extending germ band {Schwyter, 1995 #3038}. Extensive binding of Gro within these regions suggests that it also potentially has a role in *dpp* expression resulting from these elements {Spencer, 1982 #3039} {Blackman, 1991 #3040;Theisen, 2007 #3041}.

Three Dorsal binding sites identified upstream of the *tolloid* gene have been shown to be responsible for the Dorsal-mediated repression of *tolloid* in ventral regions of the embryo in the early embryo, before and during cellularization up to early stages of gastrulation. Two of these sites were shown to function as a long-range repressive element, as they silence repression of a reporter gene when fused to an adjacent promoter {Kirov, 1994 #3107}. While two of these Dorsal binding sites have been shown to be necessary and sufficient for *tolloid* repression, the mechanism of repression has not been investigated. Groucho ChIP-seq data indicates that Groucho associates strongly, in an asymmetric peak centered on the central Dorsal binding site, approximately 400 bp upstream of the *tolloid* locus (Fig. 2- C). While the peak persists through all three time windows, it’s intensity continuously decreases, with a total decrease of 2.25-fold when comparing the final to initial time window.

*Groucho associates with Dorsal-binding sites of Dorsal-activated genes*

Dorsal is also necessary for the ventral activation of a number of genes, a process that is thought to be Groucho-independent. These ventrally-activated genes include rhomboid (rho), single-minded (sim), and short gastrulation (sog) {Ip, 1992 #3042;Gonzalez-Crespo, 1993 #3043}. Loss of Gro activity was shown to result in restricted expression of these genes in 1.5 – 2 hour embryos, but did not result in significant change in expression pattern along the dorsoventral axis, so it is hypothesized that Gro is not involved in Dorsal-mediated activation of these genes.

ChIP-seq data reveals, however, that Groucho potentially plays a role in regulating expression of these genes in some portions of the embryo (Fig. 2-7). A significant Gro peak overlaps a regulatory region termed the neuroectoderm element (NEE) in 1.5 – 4 hour embryos. The area contains multiple Dorsal, Twist, and Snail binding sites, which are required for restriction of rhomboid expression to the presumptive neuroectoderm {Ip, 1992 #3042}. The Gro peak shifts towards an adjacent CRM termed the midline element (MLE) during 4 – 6.5 hours post fertilization. It is unknown which factors bind to the MLE and would be responsible for recruitment. At both timepoints, recruitment of Groucho to the regulatory region is associated with additional binding at the TSS of *rho*. This may represent a looping of the enhancer region over the 1.5 – 2 kb intervening sequence, which is depleted for Gro, potentially indicative of a repressive mechanism whereby Gro interacts with or blocks assembly of the primary transcriptional machinery.

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

Groucho binding regions are associated with fewer genes than there are total binding sites (Fig. 2-8A) due to the tendency of Groucho to localize to multiple regions around its potential targets. Half of all Groucho-associated genes have two or more Groucho peaks in relative proximity (Fig. 2-8B), with an average of 2.5 binding sites per associated gene. These peaks have median widths in the 500 – 700 bp range, indicative of point source peaks, as commonly seen for sequence-specific transcription factors, rather than the broad peaks typical of polymeric factors or histone marks (Fig. 2-9). 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 Groucho 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 promoters, UTRs, and introns*

Groucho binding is enriched close to transcription start sites (Fig. 2-10A). 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 nor enrichment. Binding within introns and 5’ UTRs is also enriched. This pattern of occupancy is somewhat surprising given 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 Gro 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. 2-10B). 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. 2-11A). Between 60 and 80% of all binding within genes occurs within introns, dependent on timepoint. Of all Groucho intronic binding sites, 40% fall within the first intron. This represents a more than 2-fold enrichment of binding preference for these introns, and is consistent 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}.

However, it is not known whether Groucho peaks arising inside genes are the result of direct recruitment of Groucho to those sites by interaction with sequence-specific transcription factors, or if Groucho is recruited to intergenic regulatory regions which are brought into contact with these introns through looping or a similar rearrangement of the local topology, resulting in immunoprecipation of these regions during ChIP-seq. However, it does appear that the interaction of Groucho with these introns is specific due to the restricted size of the intronic binding sites. The median width of intronic sites is identical to intergenic sites (403 vs 402 bp, respectively). Therefore, some property of the intron is directing Groucho to associate with specific points within. Whether that property is a protein interaction with another intron-associated factor, or a topological property of the surround chromatin, is unknown.

Motif analysis of the intronic Groucho recruitment sites identifies a small number of transcription factor biding motifs enriched at each timepoint, including some factors known to colocalize with Groucho, including twist (twi), tinman (tin), and pannier (pnr), as well as previously unassociated factors, including vielfältig (vlf) (Fig. 2-12). Enrichment of motifs are dependent on timepoint, exhibiting significant change over time. Additionally, some motifs are specific to Groucho peaks arising inside genes, such as motifs corresponding to serpent and ultraspiracle.

*Identification of Groucho Targets by Developmental Stage*

In order to incorporate our picture of Groucho binding into a framework of Groucho-mediated repression, 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 are derived from female germline clones of a Gro allele that give rise to a severely truncated and therefore non-functional Gro gene product. Analysis of Gro transcript levels across samples at each timepoint confirms overexpressing lines accumulated increased transcript levels, with the effect being greatest at the first timepoint (Fig. 2-13A); Groucho loss-of-function embryos failed to accumulate Gro transcripts to any significant degree across all timepoints. Wild-type embryos exhibit the expected pattern of initially high levels of maternally-deposited transcript, which are gradually reduced as development proceeds (Fig. 2-13B).

Clustering of RNA-seq profiles reveals the transcriptomes cluster first by timepoint, then by Groucho dosage (Fig 2-14). Groucho loss-of-function samples segregate well from wild-type and overexpression samples, while cluster discrimination between wild-type and overexpression is relatively weak, indicating that loss-of-function embryos exhibit a greater degree of transcriptome deviation from all other samples, while there are enough similarities between overexpression and wild-type embryos that they can cluster together. Groucho loss-of-function samples from the second and third timepoints cluster independently from all other samples at those two timepoints, indicating that the accumulation of differences in gene regulation has put these embryos on a highly divergent and non-viable developmental trajectory (Fig. 2-14, red box).

Principal component analysis (PCA) allows a more detailed dissection of transcriptome profile changes between Groucho dosages, and how those changes evolve over time (Fig. 2-15). PCA is a common technique used to visualize high-dimensionality data in two dimensions; linear distance between two points is directly proportional to the dissimilarity between those samples. PCA analysis reveals two sources of variance between samples: developmental stage on the x-axis, and Gro dosage on the y-axis, fitting with the major determinants of hierarchical clustering seen in the previous correlation heatmap. Comparison of the overexpression lines with the wild-type embryos shows that while these samples exhibit overall high similarity at early timepoints (upper-left cluster), overexpression samples grow increasingly distinct from wild-type over time, as can be seen by the divergence of these points from the wild-type sample (in red). Gro loss-of-function samples plot independently at all timepoints, exhibiting a strong divergence from all other samples at all timepoints.

Perturbation of Groucho levels results in the misregulation of a significant proportion of the Drosophila genome over each timespan (Fig 2-16A). 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, with the greatest effect seen in the second, 4 to 6.5 hour stage (Fig. 2-16B). Overexpression samples exhibit a smaller yet still significant proportion of differentially expressed genes, with the strongest effect seen at the final, 6.5 to 9 hour stage. Comparison of differentially expressed genes in the three Gro overexpression lines reveals significant correlation between activation or repression of genes regardless of Groucho dosage, with this effect holding across all timepoints (Fig. 2-17).

As Groucho is known to restrict the expression patterns of many developmental regulators including transcription factors, splicing factors, and signaling molecules (e.g., tailless, huckebein, zen, Sxl, dpp, etc.), 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. In order to reduce the inclusion of these secondary effects in our determination of Gro targets, we refined the list of potential Groucho targets in two ways. First, we focused on genes that exhibit a response of an opposite magnitude in the loss-of-function and one and more overexpression lines (i.e. up-regulated under conditions of lowered Gro dosage and down-regul ated under increased dosage, or vice-versa). This results in a significant restriction of the effected gene list at each timepoint (Fig. 2-18). Secondly, we narrowed this list to only those genes associated with adjacent or overlapping Groucho binding, as determined by ChIP-seq. The resulting gene list is significantly reduced, consisting of 248 genes, of which 151 are common between the 2x and 4x Gro overexpression lines (Fig 2-19 & Supplemental Table 1).

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, 2011 #1659}. 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, 2011 #2284}.

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. 2-20). 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.

*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, 1993 #2380}. Since this discovery, polymerase stalling has been found to be a ubiquitous regulatory mechanism {Conaway, 2000 #2381}, 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 {Zeitlinger, 2007 #3010}. 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) (Fig. 2-22). 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

*Groucho is recruited ubiquitiously and dynamically throughout the genome*

In our current study, we have identified thousands of novel Groucho-recruitment sites throughout the Drosophila genome. The majority of these sites are present during only one of the three timepoints analyzed, supporting the conclusion that the majority of these sites are actively participating in developmental gene regulation. The small carry-over of Gro biding from the 1.5 – 4 hr to 4 – 6.5 hr stages represents a widespread shift in Gro occupancy, consistent with the changing roles of Gro throughout development, as the availability of sequence-specific transcription factors changes across the embryo.

During the 1.5 – 4 hr stage, Gro is essential for correct determination of cell fates along the dorsal-ventral axis through cooperation with Dorsal. Groucho is recruited both within and surrounding two early ventrally-repressed genes, *zen* and *dpp*. *zen* is repressed early in a narrow stripe on the dorsal side of the embryo. The presence of a Groucho-bound peak at the transcription start site often coincides with the presence of Groucho binding upstream or inside of a gene. We hypothesize that this supports a model of repression whereby Groucho is recruited to repressive regulatory regions and precipitates a rearrangement of local chromatin, brining Gro into contact with TSS’s. Subsequent repression may be accomplished through multiple mechanisms. Gro potentially interacts with and leads to the stalling of PolII elongation, which is supported by the finding that Groucho occupancy positively correlates with stalled PolII in the developing embryo. Repression may also be initiated by the well-documented interaction of Groucho with HDAC1/Rpd3, leading to deacetylation of histones within and directly upstream of the gene body, resulting in chromatin condensation and repression. The latter mechanism of repression, via alteration of the histone mark landscape, is a potential mechanism for Groucho to act epigenetically, achieving repression that lasts after Groucho has left a locus. This is consistent with behavior seen at the *zen* locus, where Groucho occupancy is essentially gone following 4 hours of development, despite Zen remaining repressed throughout later stages of development.

Global analysis of Groucho occupancy additionally reveals that Groucho binding is strongly enriched for binding within genes, specifically within introns, with the highest enrichment exhibited in the 5’ intron of genes. Overexpression of Groucho resulted in 10 to 32% of genes bound by Gro to become repressed, depending on timepoint, reinforcing that Groucho binding within genes is one strategy common to Groucho regulation. The evolution of regulatory regions within introns is common in *Drosophila*, and in animals generally. Multiple factors, including kruppel and twist have been shown to commonly localize to intronic regions{Matyash, 2004 #3046} {Sandmann, 2007 #3048} {Zeitlinger, 2007 #3025}. The regulatory logic behind intronic cis-regulatory modules is a matter of some debate, as there are significant energetic costs associated with intron maintenance during replication, transcription, and splicing, as well as a regulatory cost in terms of a longer lag-time between transcriptional activation and mature mRNA formation {Yenerall, 2011 #3051}. Consistent with this hypothesis, developmentally-regulated genes known to exhibit promoter-proximal pausing of RNA PolII have been shown to have a higher frequency of intron loss but not overall shorter introns {Jiang, 2014 #3052}. One potential explanation of the regulatory rational that gives rise to intragenic repressor binding comes from the observation that repression arising from Snail binding in distant CRMs introduces a lag before repression becomes complete, due to the inability of the repressor to affect currently elongating polymerases {Bothma, 2011 #2304}. Due to the relatively slow rate of progression of PolII (~ 1.1 to 1.5 kb per min in *Drosophila)* {Ardehali, 2009 #3053}, this lag time can become significant, especially under developmental contexts in which temporal control of repression can be as important as activation. While microRNAs are known to dampen this effect in some contexts {Biemar, 2005 #3054}, Groucho-mediated repression initiated by binding intragenically could potentially be another method to achieve a similar end.

## References