# **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 to numerous regulatory regions 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’s 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 {Turki-Judeh, 2012 #2966}.

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 us to thoroughly investigate the requirement for spreading in Gro-mediated repression. We find that while Groucho is capable of spreading over long regions of chromatin, this spreading appears to be an uncommon feature of repression, with the majority of Groucho binding occurring in discreet peaks characteristic of association with site-specific transcription factors. However, these discrete peaks often cluster over longer stretches of chromatin, potentially indicative of looped interactions, or a similar topological rearrangement, between distant regions of chromatin.

The accurate assignment of a regulatory region, or even an individual 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 the definition of a high-confidence set of Gro target genes across developmental stages, thus enabling a more thorough characterization of the role of Gro during early development and a significant refinement of the factor’s influence on the developmentally-regulated gene network. The analysis to be presented here shows that Groucho targets are enriched for numerous transcription factors, confirming its role as a pioneering factor in the establishment of developmental 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 dynamically recruited to thousands of sites throughout embryonic development*

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. 2-2A). 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 identified 79% of the modENCODE ChIP-chip peaks. An additional 81% of our identified Gro binding sites are novel and are not represented in the data generated by the modENCODE consortium. Comparison of our ChIP-seq data with modENCODE Groucho ChIP-chip data generated from white pre-pupae, a later stage of development approximately 120 hours post-fertilization encompassing the early stages of pupal formation and metamorphosis, also shows a significant overlap (Fig. 2-2B). A large fraction of embryonic and pre-pupal binding sites are unique to each stage, consistent with the distinct roles of Groucho-mediated repression during pupal development, when Gro is primarily involved in imaginal disc patterning {de Celis, 1995 #3171}. Approximately a third of embryonic peaks are retained to some extent in this later stage, indicating Gro may be utilized in the regulation of a subset of common genes throughout multiple developmental stages.

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. 2-3A). Peaks overlapping input peaks were removed, as they are assumed to arise from erroneous read alignment due to abundant or repetitive sequences. Groucho recruitment sites are 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 2-3B).

Groucho occupancy is highly dynamic and reversible. Approximately 75% of all Groucho binding sites are unique to a single timepoint. The majority of the sites established during time window 1 that persist into time window 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.

Genome-wide analyses of transcription factor binding in the *Drosophila* embryo has revealed thousands of HOT (Highly Occupied Target) regions to which large numbers of unrelated factors bind concurrently {Consortium, 2010 #759}. While the cause and regulatory ramifications of these highly-occupied regions remain to be fully explored, they appear to be widespread in eukaryotes, persistent between cell types and developmental stages, and are often located in areas of active transcription {Moorman, 2006 #3119}. Some factors can be recruited to HOT regions independently from their ability to bind and recognize DNA sequence {Li, 2008 #2374}. Owing to this, and the the large number of Groucho-interacting proteins that either bind DNA directory or are otherwise recruited to chromatin, we expected that a significant fraction of Groucho binding sites would localize to these areas (Fig. 2-13). We observe that while the total percentage of Groucho regions that overlap a HOT zone is largely invariant between time points, Groucho in the 1.5 – 4 hr embryo preferentially localizes to regions with a higher HOTness (i.e. greater numbers of occupying factors), while 6.5 – 9 hr Groucho binding is enriched for overlap with lower HOTness regions.

The clearest theory on the function of the origin of these HOT regions, supported by *in vivo* and computational studies, is that many transcription factors are maintained at sufficiently high nuclear concentrations such that these factors saturate high-affinity binding sites, and as a result also bind to low and intermediate affinity sites in areas of high DNA accessibility {Li, 2008 #2374} {Kaplan, 2011 #3172} {Li, 2011 #3173}. As Groucho is known to increase nucleosome density and reduce DNA accessibility {Sekiya, 2007, r08904} {Winkler, 2010, r07182}, widespread recruitment to these sites of known high DNA accessibility presents a

*Groucho tends to bind in spatially-restricted clusters at promoters and inside genes*

Choosing the nearest or overlapping gene as a potential Groucho-regulated gene, we see that there are significantly fewer Groucho-associated genes than there are Groucho binding regions (Fig. 2-8A), due to the tendency of Groucho to localize to multiple discrete regions around its potential targets. Half of all Groucho-associated genes predicted in this fashion have two or more Groucho peaks in relative proximity (Fig. 2-8B), with an average of 2.5 binding sites per associated gene (compared to an expected value of 1.5 binding sites per gene, *p* < 10-10 via Monte-Carlo simulation). These peaks have median widths in the 500 – 700 bp range, indicative of point source peaks, as commonly seen for sequence-specific transcription factors {Ho, 2011 #3117}, rather than the broad peaks typical of either highly polymeric factors or histone marks (Fig. 2-9). Interesting, *in vitro* studies have shown that Grg3/repressor complexes bind to and protect DNA from nuclease activity over the span of 3 to 4 nucleosomes {Sekiya, 2007 #1658}, corresponding to 600 – 800 basepairs of protection, consistent with our observed mean peak width.

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, Groucho may be capable of spreading over relatively large regions of the genome. However, this does not appear to be a widespread mode of chromatin association. Average Groucho peak widths increase slightly 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 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 recruitment overlapping or closely adjacent to start sites is somewhat unexpected given extensive evidence that Groucho is a long-range repressor, often bound tens of kilobases away of a regulatory target {Dubnicoff, 1997 #2366} {Barolo, 1997 #2365}. 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 smaller fraction of binding overlaps start sites (3 – 10%), and intergenic binding is nearly evenly split between binding upstream and downstream of the nearest feature.

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. 2-11). 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, and therefore predicted to be enriched for regulatory elements {Bradnam, 2008 #3034}. Dorsal-ventral patterning genes in *Drosophila* are documented to be particularly enriched for cryptic, remote 5’ exons and, consequently, long initial introns potentially harboring regulatory features, with over 10% of protein-coding DV genes exhibiting this feature {Biemar, 2006 #3116}.

Motif analysis of Groucho recruitment sites identifies a small number of transcription factor biding motifs enriched at each timepoint, including several factors known to interact with Groucho, including Ventral nervous syndrome defective (vnd), Sloppy paired 1 (slp1), Hairy (h), Huckebein (hkb), and Brinker (brk) (Fig. 2-12). Enrichment of motifs varies by timepoint as well as by the location of the Groucho binding site. The majority of factors analyzed exhibit stronger enrichment for Groucho sites within genes, which can be explained by a smaller group of regulators being responsible for Groucho recruitment with genes, or less low-affinity and less specific binding of Groucho in these regions.

*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 *zerknullt* (*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 well-characterized 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 to regions surrounding the VRR, however Gro density is comparatively weak within the VRR region itself (Fig. 2-4A). Stronger Gro signal is seen both directly upstream (relative to *zen*) of the VRR, 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 stage, 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. While *zen* becomes universally repressed following 4 hours of development, Gro does not appear to remain localized the region, indicating that either Groucho establishes a repressive state that persists into the adult fly, or that another unknown factor relieves Gro of its regulatory obligation.

Dorsal is additionally responsible for ventral repression of *decapentaplegic* (*dpp*) in early embryos (1.5 – 2 hours post fertilization) through the recruitment of Gro, and 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 gene’s second intron {Huang, 1993 #3037}. Our ChIP-seq data confirms extensive Gro recruitment to this site (Fig. 2-4B), also indicating 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 originating 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 early embryo, before and during cellularization and up to early stages of gastrulation (3 hours post-fertilization). 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 thoroughly 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. 2-4C). 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. While Groucho occupancy decreases as development progresses, *tolloid* expression decreases as well, falling off sharply after 2-4 hours of development, and decreasing to negligible levels by 12 hours post-fertilization.

The three genes discussed above are well-characterized targets of Dorsal and Groucho. All three targets are repressed in ventral portions of the embryo by Groucho through its interaction with Dorsal, all at roughly the same developmental stage. And yet, temporally-discrete ChIP-seq analysis of these genes reveals strikingly different patterns of Groucho recruitment and retention. Groucho associates with all three genes during the developmental time frame when the gene is being actively expressed in some portion of the embryo, and, for *zen* and *tll*, Groucho binding ceases or significantly decreases when these genes cease being actively expressed in dorsal regions of the embryo. Conversely, *dpp* continues to be expressed at moderate levels throughout embryonic development, and retains significant levels of bound Groucho in some regions of the embryo. Together, this supports a model of Groucho activity whereby Groucho is recruited specifically to genes to spatially restrict expression, but continued presence is not necessary to maintain repression of universally silenced genes.

At each gene, Gro appears to be specifically recruited to the known Dorsal binding sites, and in each case significant Groucho occupancy is observed overlapping the target’s promoter and transcriptional start site. In the case of *tll*, Gro is recruited in a wide peak which overlaps both the Dorsal binding site and the TSS. In the case of *zen* and *dpp,* Gro is recruited to Dorsal sites either further upstream of the gene or within the gene body, with additional discrete peaks appearing at the TSS. These discrete peaks are potentially indicative of looping by Groucho from the recruitment site into the promoter region and provide additional evidence against the hypothesis that Gro spreads across large regions of chromatin. Groucho association with promoter regions has potential implications for the mechanism of Grouch-mediated repression and provides a plausible explanation for the observation that Groucho is capable of long-range repression, i.e. counteracting the activating potential of additional transcription factors bound to the same genomic region. The genome-wide prevalence of these patterns is analyzed further in subsequent sections.

*Groucho localizes extensively to the Dorsal-binding sites of both Dorsal-activated and –repressed genes*

In addition to repressing multiple genes in the ventral portion of the embryo, Dorsal can activate genes in both ventral and ventrolateral regions of the embryo in a context-dependent manner. The transition of Dorsal from an activator to a repressor is thought primarily to be determined by the presence of adjacent binding sites for additional factors, such as Deadringer and Cut, that facilitate the association of Groucho with Dorsal, resulting in Groucho-mediated long-range repression {Valentine, 1998 #3036}. The necessity of these factors in generating a stable Dorsal/Groucho interaction is thought to arise from the relatively low binding affinity of Groucho for Dorsal, when compared to factors to which Groucho binds without requiring assistance, such as Engrailed or Brinker {Ratnaparkhi, 2006 #3108}. Due to the inherent weakness of the Dorsal/Groucho interaction, it is not suspected that Groucho would ubiquitously colocalize with Dorsal, and would instead only associate at those loci at which Dorsal functions as a repressor. Our Groucho ChIP-seq data, however, shows that that is not strictly the case.

In ventral regions of the embryo, Dorsal serves to activate several genes, the two most well-studied being *twist* and *snail,* two transcription factors essential to the specification of the presumptive mesoderm and later coordinate invagination of this region to form the mesodermal germ layer {Thisse, 1987 #3109} {Ip, 1992 #3110}. Dorsal activates *twist* through binding to a Ventral Activation Region (VAR) {Pan, 1991 #3111}, and recruitment of the co-activator dCBP {Akimaru, 1997 #3112}. Twist then serves as a co-activator to initiate expression of *snail* in ventral regions of the embryo {Ip, 1992 #3110}. No role for Groucho has been identified in the regulation of either gene.

Temporally-discrete profiling of Groucho occupancy reveals that Groucho may play an unexplored role in regulation of these two genes. Two cis-regulatory regions have been identified upstream of *snail*, either of which is sufficient for Dorsal-mediated activation of the gene in ventral regions of the early (2 – 3 hr) embryo {Ip, 1992 #3110}, leading to the hypothesis that the secondary enhancer, referred to as a shadow enhancer, is involved in fine-tuning *snail* expression, or potentially making expression more robust to stochastic fluctuations in transcription factor availability {Perry, 2010 #385}. Both Dorsal and Twist were found to bind extensively in both enhancer regions in 2-4 hr embryos {Zeitlinger, 2007 #3025}. Unexpectedly, Groucho also occupies both regions in 1.5 – 4 hr embryos, as well as multiple additional sites within the 5kb span between enhancers (Fig. 2-5A). While the two enhancers are thought to be functionally redundant with regards to Dorsal/Twist regulation of *snail*, they differ in their ability to recruit Groucho. Both sites exhibit a strong Groucho peak, which is lost in the primary enhancer by the 4 - 6.5 hr stage but retained by the shadow enhancer. These peaks may represent a role for Groucho in repressing *snail* repression in dorsal and lateral regions of the embryo, potentially through an interaction with Brinker, a Groucho-interacting repressor involved in Dpp-signaling and active in dorsolateral and lateral portions of the embryo {Zhang, 2001 #3099}. This is indicative of a role for Groucho in limiting spurious activation of snail by the low concentrations of nuclear Dorsal in these regions.

In contrast, recruitment of Groucho to the *twist* locus is relatively weak. Dorsal binds within the ventral activation region (VAR) directly upstream of *twist,* where it serves to activate gene expression via the cooperation of the co-activator dCBP. A small yet significant Gro peak is present within this region during the first time window, but disappears by later stages (Fig. 2-5B). While Groucho may be involved in repressing *snail* in dorsal and dorsolateral regions of the embryo, it appears *twist* repression is initiated or maintained by another, unknown, mechanism.

Dorsal is also necessary for the activation of a number of genes in ventrolateral regions of the embryo, 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 {Dubnicoff, 1997 #2366}.

ChIP-seq data reveals, however, that Groucho potentially plays a role in regulating expression of these genes in some portions of the embryo (Fig. 2-6). A significant Gro peak overlaps a regulatory region termed the neuroectoderm element (NEE) upstream of *rho* 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, again indicative of a repressive mechanism whereby Gro interacts with or blocks assembly of the primary transcriptional machinery.

Dorsal binding sites can be subdivided into three classes dependent on the resulting expression pattern of the regulated gene {Biemar, 2006 #5} {Zeitlinger, 2007 #3025}. This variance is established via the establishment of Dorsal as an activator or repressor as well as the strength of Dorsal binding, processes poorly understood, but thought to be controlled by the number and patterning of Dorsal binding sites, as well as adjacent recruitment of additional transcription factors. This tightly-controlled definition of Dorsal activity is critical to the correct interpretation of the Dorsal gradient present along the dorsoventral axis of the early embryo. Class I regulatory regions result in gene expression in the most ventral regions of the embryo (presumptive mesoderm), indicative of Dorsal mediated activation. Class II regions generate expression in ventrolateral regions (dorsal neuroectoderm), an area with intermediate levels of nuclear Dorsal. Class III regions result in expression in the dorsal ectoderm, which has the lowest Dorsal concentrations. Class III activation is thought to arise from Dorsal-mediated repression in ventral and ventrolateral regions. Groucho is found to bind to all three types of sites, with overlap strongest during the second time window, 4 – 6.5 hours (Fig. 2-7A). Association with Class I sites is the lowest by timepoint. No single class of Dorsal site is significantly enriched over the others, indicating that Groucho binds to Dorsal more frequently than previously surmised, even in situations (such as Class I sites) where Dorsal is activating transcription.

In addition to DV patterning determinants, Dorsal has been found to participate in additional patterning pathways, including the regulation of multiple types of genes involved in segmentation, including gap, pair-rule, segment polarity genes, and homeotic genes involved in limb specification. The nature of Dorsal’s role in these processes remains largely uninvestigated. A significant number of these sites appear to also recruit Groucho in 1.5 – 6.5 hr embryos (Fig. 2-7B), lending credence to Dorsal potentially participating in the regulation of these genes via interaction with Groucho. That Groucho would be involved is in anterioposterior patterning is not surprising, as numerous examples of Groucho’s roles in segmentation definition via interaction with Engrailed and Hairy family proteins have been described previously {Jiménez, 1997 #264}.

*Groucho colocalizes with Dorsal to areas of high transcription factor density in the very early embryo*

To further probe the nature of Groucho’s association with these multiple factor-bound regions, we calculated the combinatorial overlap of each Groucho binding segment with the binding patterns of 25 additional transcription factors derived from 2 – 4 hr embryos {MacArthur, 2009 #6}. A factor heatmap of the hierarchically clustered Groucho binding regions reveals two major classes of Groucho binding site. The first class is characterized by extensive, nearly uniform, overlap with six factors: Dorsal, Dichaete, Medea, Twist, Daughterless, and Kruppel, and lesser degrees of overlap with one or more additional assayed factor (Fig. 2-14). While Dorsal is a well-studied Groucho-interacting protein, the degree to which Groucho colocalizes with Dorsal is surprising, given that there are at minimum thirteen other factors capable of recruiting Groucho in processes thought to be Dorsal-independent {Mannervik, 2014 #2280}. The second major class of Groucho binding site, comprising ~25% of Groucho sites in the early embryo, lacks overlap with any of the assayed transcription factors. There are at least two feasible explanations for this observation. Given that overlap was only calculated against 25 of the estimated ~700 transcription factors contained in the *Drosophila* genome {Adams, 2000 #3120}, there could exist factors, or entire classes of factors, to which Groucho is being recruited that have yet to be identified. Additionally, some of these sites may represent recruitment of Groucho to chromatin in a manner not dependent on additional factors, for example through interaction with histones.

*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). Overexpression of a deletion variant of Groucho lacking the SP domain was found to result in faulty targeting and ectopic repression of multiple non-Groucho target genes {Turki-Judeh, 2012 #2966}, a trend that we sought to investigate on a genome-wide scale. Additionally, we analyzed the transcriptome of embryos lacking maternally-contributed functional Groucho. These embryos are derived from female chimeric germline clones homozygous for *groMB36*, a lethalallele that introduces an ectopic splice site near the 5’ end of *gro* {Jennings, 2007 #2990}. The resulting transcript codes the initial 12 amino acids of Groucho followed by ~100 amino acids derived from frameshifted sequence. The allele produces no detectable Groucho protein, and severely decreased levels of transcript, presumably due to nonsense-mediated mRNA decay. 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. 2-16A). This excess transcript is partially cleared from the embryo by later timepoints, but does not fully return to wild-type levels over the time span analyzed. 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. 2-16B).

Clustering of RNA-seq profiles by similarity reveals the transcriptomes cluster first by timepoint, then by Groucho dosage (Fig 2-17). 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, indicative that accumulated differences in gene expression have put these embryos on a highly divergent and non-viable developmental trajectory (Fig. 2-17, 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. 2-18). 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 2-19A). The Groucho loss-of-function phenotype was more severe than that obtained from overexpression, with over 10% of expressed genes exhibiting significant changes in expression level at each timepoint, with the greatest effect seen in the second, 4 to 6.5 hour stage (Fig. 2-19B). Overexpression samples exhibit a smaller yet still significant proportion of differentially expressed genes, with between 2 and 16% of the expressed genome undergoing differential expression, 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. 2-20).

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-regulated under increased dosage, or vice-versa). This results in a significant restriction of the effected gene list at each timepoint (Fig. 2-21). 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 two full-length Gro overexpression lines (Fig 2-22 & Supplemental Table 1).

To further explore the relationship of Groucho occupancy and regulation, we employed a scoring algorithm to quantify the predictive power of Groucho binding to changes in expression. To do so, we modified a procedure successfully utilized to predict the targets of CBP, a coactivator that cooperates with Dorsal to activate gene expression in the early embryo, using CBP ChIP-seq data and measurement of a mutant CBP transcriptome {Holmqvist, 2012 #3115}. On a per-gene basis, a “Groucho occupancy score” was calculated taking into account the number, size, and positioning of any Groucho peaks. Operating under a progressively relaxing score cutoff, the number of genes captured with scores above said cutoff that are up- or down-regulated upon Groucho level perturbation were counted (Fig. 2-24). The inflection point of the resulting response curves can than be used as an empirically-derived threshold for classifying Groucho target genes.

We find that the changes in gene expression resulting from Groucho overexpression are significantly more predictive of regulation than changes resulting from loss of Groucho activity (Fig. 2-24B/C), i.e. that Groucho binding to or near a gene more often indicates that that gene can be repressed by increased Groucho availability. Very few up-regulated genes are captured by the response curve in overexpressing lines, especially at early timepoints. In *groMB36* embryos, a very slight enrichment of derepressed genes is evident during the first two time spans with clear inflection points (Fig. 2-24A).

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. 2-25). 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*

Promoter-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 in higher eukaryotes {Conaway, 2000 #2381}, with strong peaks of PolII present in the promoter regions of a diverse array of genes throughout the *Drosophila* genome. The majority of development regulators in humans are regulated in some capacity post-initiation {Guenther, 2007 #3118}, as is a large fraction of the *Drosophila* developmental genome {Zeitlinger, 2007 #3010}.

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. 2-26). 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

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