Chapter 6

Global quantitative proteomics reveals novel factors in the ecdysone signaling pathway in *Drosophila melanogaster*

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Abstract

The ecdysone signaling pathway plays a major role in various developmental transitions in insects. Recent advances in the understanding of ecdysone action have relied to a large extent on the application of molecular genetic tools in *Drosophila*. Here, we used a comprehensive quantitative SILAC mass spectrometry-based approach to study the global, dynamic proteome of a *Drosophila* cell line to investigate how hormonal signals are transduced into specific cellular responses. Global proteome data after ecdysone treatment after various time points were then integrated with transcriptome data. We observed a substantial overlap in terms of affected targets between the dynamic proteome and transcriptome, although there were some clear differences in timing effects. Also, downregulation of several specific mRNAs did not always correlate with downregulation of their corresponding protein counterparts and in some cases there was no correlation between transcriptome and proteome dynamics whatsoever. In addition, we performed a comprehensive interactome analysis of EcR, the major target of ecdysone. Proteins co-purified with EcR include factors involved in transcription, chromatin remodeling, ecdysone signaling, ecdysone biosynthesis and other signaling pathways. Novel ecdysone-responsive proteins identified in this study might link previously unknown proteins to the ecdysone signaling pathway and might be novel targets for developmental studies. To our knowledge, this is the first time that ecdysone signaling is studied by global quantitative proteomics.

Introduction

Drosophila melanogaster belongs to the phylum Arthropoda of the animal kingdom, which includes insects, crustaceans, mites, arachnids, scorpions and myriapods. The rigid exoskeleton of these invertebrate animals inhibits growth, so arthropods replace it periodically by molting. Insect molting hormones or ecdysteroids, such as ecdysone, are key regulators of major post-embryonic events, including the larval-to-larval molts and the larval-to-pupal metamorphic transformation (Riddiford 1993). Ecdysone is a prohormone of the major insect molting hormone 20-hydroxyecdysone (20E; hereafter simply referred to as 'ecdysone'), which is secreted from the prothoracic glands where it is produced by the enzymatic conversion of cholesterol. The glands are stimulated to undergo steroidogenesis in discrete and periodic surges and this is reflected in peaks of the active ecdysone observed in larvae and pupae, all precisely generated by either increased rates of steroidogenesis or alternative metabolic processing (Warren et al. 2006). In the larvae, the circulating prohormone secreted by the prothoracic gland is only converted to the active hormone in target tissues (Talbot, Swyryd, and Hogness 1993).

Ecdysone is a substrate for the ecdysone receptor, which is a non-covalent heterodimer of two proteins, Ecdysone Receptor (EcR) and ultraspiracle (USP). These nuclear hormone receptor proteins are the *Drosophila* orthologs of the mammalian farnesoid receptor (FXR) and retinoid X receptor (RXR) proteins, respectively. EcR interacts with USP to bind to various ecdysone response elements (EcREs) to transactivate several target genes (Yao et al. 1993) (Figure 1). EcR and USP are the best known dimerization partners which activate ecdysone-responsive genes; however, there is more variation. The *EcR* gene itself already encodes 3 different isoforms; EcR-A, EcR-B1 and EcR-B2 (Koelle et al. 1991), which share common DNA and ligand binding domains, but differ in their N-terminal sequences, which are involved in transcriptional activation. Besides the existence of multiple isoforms of EcR, different EcR binding partners have been described. Recently, it was found that EcR can dimerize with DHR38 (Zoglowek et al. 2012). In addition, there is genetic evidence that USP is not required for the ecdysone-dependent activation of the larval glue genes (Costantino et al. 2008). The ability of nuclear receptors to form multiple, different heterodimers suggests that their role in regulatory events may be more complex than previously anticipated. Additional ecdysone-sensitive nuclear receptor proteins have yet to be elucidated though.

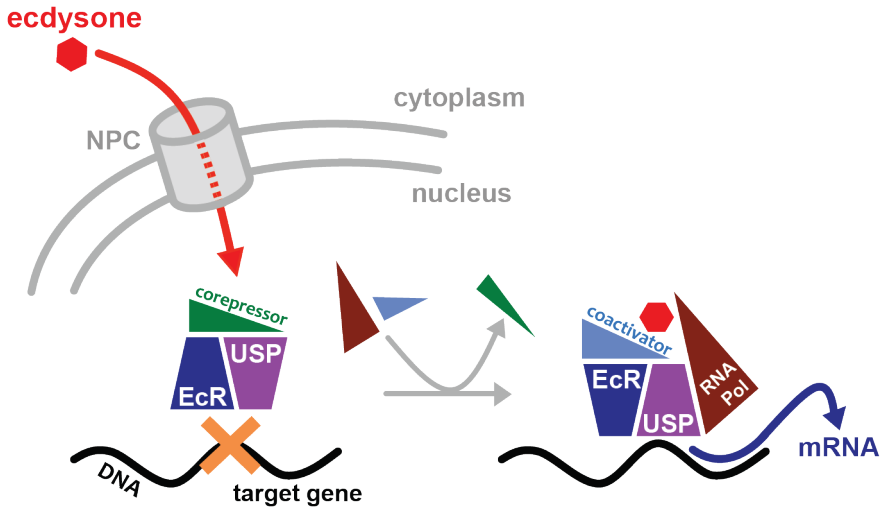

Figure 1. Simplified representation of the mode of action of ecdysone in *Drosophila*. When ecdysone enters the nucleus it binds to the heterodimer EcR/USP, which subsequently recruits coactivators and RNA Pol II to transcribe target genes.

The role of ecdysone in gene regulation was elucidated based on the identification of rapid and delayed puff induction upon hormone treatment of cultured salivary glands (Clever and Karlson 1960). Puff induction can be interpreted as local transcriptional activity and, based on the timing, early and late puffs can be identified. Later, it was experimentally shown that early puffs were directly induced by ecdysone (by Clever in *Chironomus* (Clever and Karlson 1960) and by Ashburner in *Drosophila* (Ashburner 1974) and are direct targets of the ecdysone-bound receptor, while the formation of late puffs was an indirect effect because only the latter were affected by the use of protein synthesis inhibitors. This concept is referred to as the 'Ashburner model' (Ashburner 1974, Ashburner 1972). Several ecdysone responsive genes have been identified; examples include *E74, E75* and *Broad-Complex* (*BR-C*),which encode transcription factors that, in turn, drive the expression of late genes, while downregulating the expression of early genes (Andres and Thummel 1992). The early genes have been shown, like the early puffs, to be directly and transiently induced by ecdysone (Felix D. Karim and Thummel 1991; F D Karim and Thummel 1992) and mutant analysis has demonstrated that E74 and BR-C functions are essential for proper entry into metamorphosis (Fletcher et al. 1995; Kiss et al. 1988; Restifo and White 1992). Early gene *E63-1* encodes a calcium-binding protein which is closely related to calmodulin (Thummel 2002). Besides early and late genes, a class of 'early-late' genes was discovered (Stone and Thummel 1993). These genes require the ecdysone-bound EcR/USP heterodimer, but besides that, do also require an early gene product for optimal transcription induction. Examples of early-late genes are the nuclear receptor proteins DHR3 and DHR4, which repress the early genes *BR-C*, *E74* and *E75*. Both DHR3 and DHR4 are required for *FTZ transcription factor 1* (*ftz-f1*) expression in mid-prepupae (King-Jones et al. 2005; Lam, Jiang, and Thummel 1997), which strongly suggests that these two factors act in concert to regulate the early genes and *ftz-f1*. Mutant and knock-down studies have revealed additional proteins involved in ecdysone signaling, for instance WOC (without children) (Wismar et al. 2000), MLD (molding defective) (Neubueser et al. 2005) and DRE4 (Sliter and Gilbert 1992). Several genome-wide (microarray) analyses of ecdysone signaling have been published, for instance on specific *Drosophila* organs (Beckstead, Lam, and Thummel 2005). Another study revealed large differences between cultured Kc cells and salivary glands with regard to their genome-wide transcriptional response to the hormone (Gonsalves et al. 2011). Experiments with conditional mutants have suggested that separable transcriptional responses among ecdysone, the pre-hormone receptor and the post-hormone receptor occur (Davis and Li 2013). However, many aspects of especially early-late and late phases downstream of the ecdysone signaling cascade are still unknown and the roles of many previously identified ecdysone responsive factors, as well as yet unknown factors remain to be elucidated.

We are studying the regulation of *Drosophila* metamorphosis by the steroid hormone ecdysone as a model system for understanding how hormonal signals are transduced into

specific cellular responses, in particular those at the proteome level, and try to link these to developmental pathways. In this study, we used a comprehensive quantitative (SILAC) mass spectrometry-based approach to study the dynamic proteome in an attempt to identify novel proteins that respond to ecdysone stimulation in *Drosophila*. Since gene expression involves the transcription, translation and turnover of mRNAs and proteins, we have monitored how ecdysone treatment affects protein and mRNA abundances in time. This strategy provided a detailed, integrative analysis of proteome and transcriptome composition changes in response to cellular perturbations, and of the dynamic interplay of mRNA and proteins. To our knowledge, this is the first time that ecdysone signaling is studied by quantitative proteomic methods. Previous studies in *Drosophila* and several other species where mRNA and protein levels were compared to provide a quantitative description of gene expression concluded that the correlation is weak (*e.g.*, (Ghazalpour et al. 2011; Laurent et al. 2010; Maier et al. 2011; Schwanhüusser et al. 2011)). One reason for this could be that mRNA and protein levels result from complex coupled processes of synthesis and degradation. In addition, since variation of ecdysone receptor dimerization appears to be important for proper and specific regulation of ecdysone-responsive gene products during different stages of development, we used a comprehensive experimental approach for the identification of novel interactors of endogenous EcR. Novel ecdysone-responsive proteins identified in this study might link previously unknown proteins to the ecdysone signaling pathway and might be targets for further developmental studies.

Results and discussion

Proteome and transcriptome expression analysis

We set out by verifying the effect of ecdysone treatment on two different *Drosophila* cell lines, Kc and S2, by monitoring RNA expression levels of two known ecdysone responders by quantitative real-time RT-PCR. The effect on Kc cells appeared to be larger and as it was suspected that this would therefore result in larger changes at the proteome level we decided to continue all experiments with Kc cells (Suppl Figure 1; data for S2 cells not shown). Since no commercial SILAC medium is available for insect cell lines, medium depleted for arginine and lysine was prepared based on both the composition of commercially available Schneider's insect medium and protocols in the literature (Bonaldi et al. 2008). Details of the SILAC medium formula that was used in this study are given in Suppl Table 1. Cells grown in this medium formula did not show any deviations in size, shape, or growth rate, as compared to cells grown in conventional medium. In order to avoid differences in characteristics of cells grown in light and heavy medium, all experiments were performed in a forward and reverse fashion and only those

identification hits that show consistent H:L ratios in the duplicate experiments were taken into account for further downstream analysis.

We first questioned whether ecdysone treatment of cells results in measurable changes at the level of the final effectors in the cell, *i.e.* its global proteome. Next, we questioned whether ecdysone signaling has similar effects on protein expression as it has on RNA expression in terms of the amount of up- and downregulation and timing. In the SILAC global proteome screen, we identified and quantified in total 5,748 proteins over 6 different time points in the range of 0 – 96 h after hormone induction of Kc cells (Suppl Table 2). These time points were chosen based on transcriptomics studies in the literature, which indicate that early responses already take place within several hours after ecdysone supplement to the cell. Extended time points were chosen to include effects of the putative delay between expression at the proteome level expression versus the transcriptome level. Quantitative proteomics analysis revealed that the abundances of the far majority of proteins remain unchanged (Figure 2): the scatter plots of H:L ratios of forward and reverse experiments after treatment of Kc cells with ecdysone for the indicated incubation times show a narrow distribution of data points around zero. However, although the far majority of protein abundances remain unchanged, there is a small subset of proteins that are up- or downregulated already at early time points. Among this small set of proteins are ecdysone induced protein 71 (Eip71CD or Eip28/29), eater, CG18765, mus309, Regeneration (rgn), glycine N-methyltransferase (CG6188) and BR-C (indicated in red in Figure 2 in the early time point plots). Eip71CD is a repair enzyme for proteins that have been inactivated by oxidation (Abhilash Kumar et al. 2002) and is a known ecdysone early responder, while eater is a major phagocytic receptor for a broad range of bacterial pathogens in Drosophila (Kocks et al. 2005). CG18765 is a protein of unknown function with no apparent ortholog in Mammalia, mus309 (Bloom syndrome protein homolog, Blm) is involved in DNA replication and repair (Adams, McVey, and Sekelsky 2003; Andersen et al. 2011) and Rgn controls the timing, the site and the size of blastema formation (McClure, Sustar, and Schubiger 2008). BR-C has also previously been described as an early ecdysone responsive gene. It is required for puffing and transcription of salivary gland late genes during metamorphosis (Crossgrove et al. 1996) and has been associated to a broad range of other cellular events such as oogenesis and organ development. It positively regulates transcription from the RNA polymerase II (RNA Pol II) promoter.

At early time points, BR-C is upregulated at the protein level, but after longer time points the increase levels off (Figure 3A). A similar effect is observed at the mRNA level (see Figure 5D). At later time points, the set of affected proteins grows, but is still small fraction of the total (measurable) proteome. The consistent H:L ratios in the forward and reverse SILAC experiments respectively (Figure 3B), are in agreement with the behavior of BR-C behavior of BR-C on immunoblots after ecdysone stimulus as compared to mock samples (Figure 3D). BR-C was identified by only one tryptic peptide

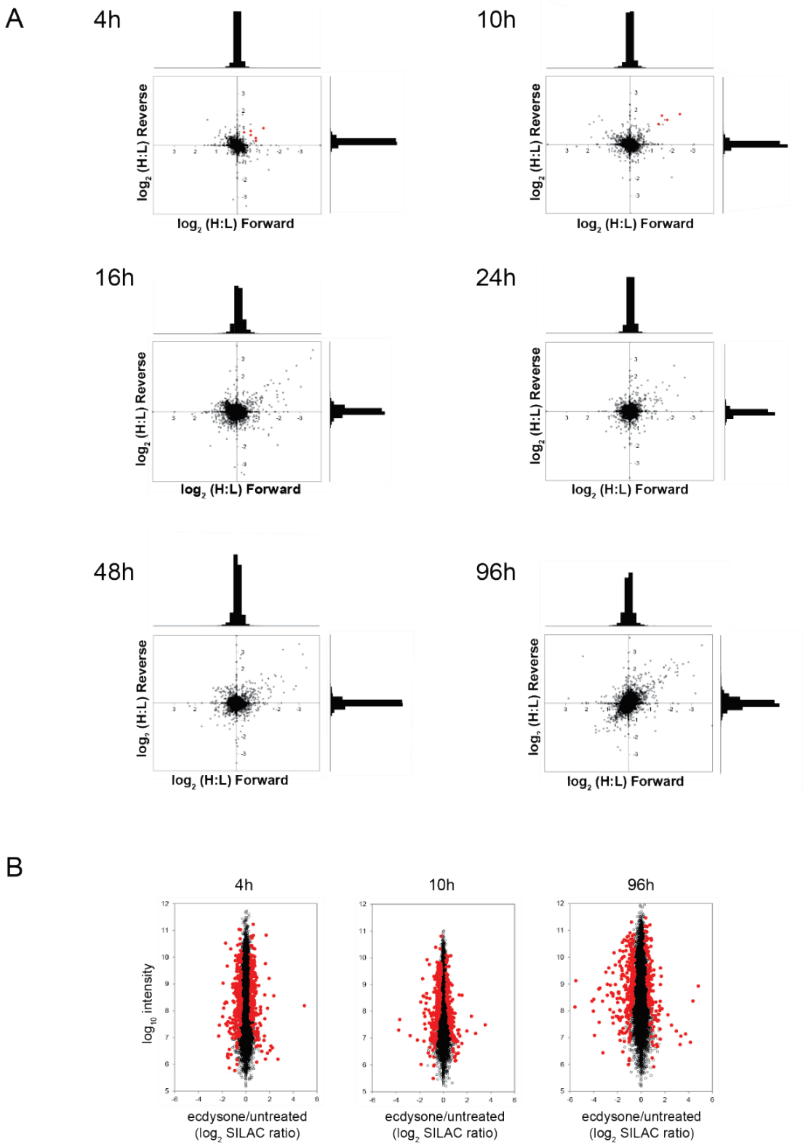

Figure 2. A) Scatter plots of H:L ratios of forward and reverse experiments obtained from SILAC MS assays after ecdysone treatment for the indicated incubation times. Although the far majority of protein abundances remain unchanged after this cellular perturbation, there is a small subset of proteins that show ratios deviating from 1 (0 at a $\log_2$ scale), *i.e.* proteins which are up- or downregulated, especially at later time points. **B)** Significant outliers (p-value < 0.05) in the SILAC data sets as determined by the Significance B protocol in Perseus for separately treated forward and reverse experiments. Significant outlier analysis was done prior to filtering for consistency of forward and reverse ratios as the additional selection criterion.

in our assay, but manual inspection of the fragmentation spectrum of this peptide firmly confirms its identity (Figure 3C). The quantitative analysis of BR-C shows the proof of concept of the SILAC mass spectrometry setup used in this study.

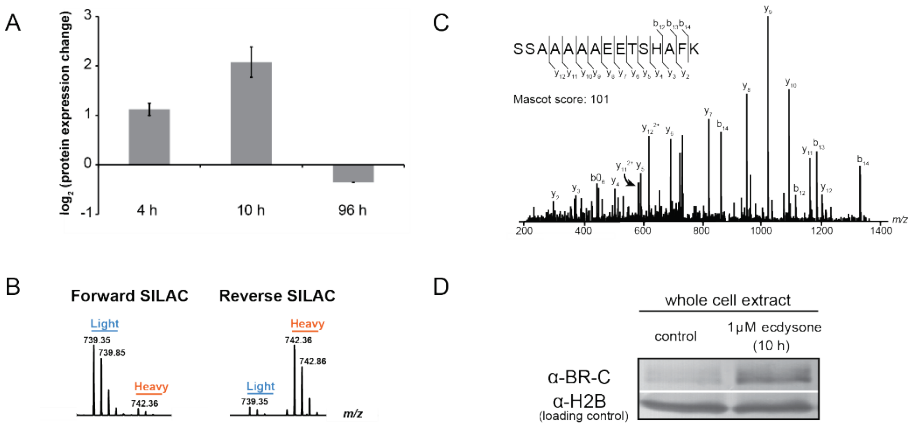

Figure 3. **Detailed SILAC data for BR-C.** A) Relative protein abundance changes after ecdysone treatment for the indicated time periods. After relative short incubation times, BR-C is upregulated, while after much longer incubation times the abundance returns to pre-treatment values or even lower. Error bars are the SD of two replicates. B) Zoom spectra from forward and reverse SILAC experiments representing a BR-C tryptic peptide in its light (blue) and heavy (orange) form. The H:L ratios in the forward and reverse (after label swap) are consistent and indicate an upregulation of BR-C after 10 h. C) Fragmentation spectrum of the BR-C tryptic peptide (m/z of [M+2H]2+: 739.34) confirms its identity; b- and y-ions are indicated. D) Immunoblot using specific antibodies against BR-C on mock cells and cells that were treated with ecdysone for 16 h. The upregulation of BR-C after treatment is in agreement with the relative protein abundance data from the SILAC screen. H2B was used as the input control.

All downstream data analysis hereafter was performed using a subset of the SILAC time points described above, *i.e.* fast, intermediate and long treatment (4, 10 and 96 h, respectively), for technical reasons. The protein numbers in terms of identification and relative quantitation for the data set derived from the Q Exactive based workflow were considerably higher and resulted therefore in the highest-quality data set. In total, 4,503 hits of the SILAC proteome data set and the RNA sequencing (RNAseq) data set were overlapping. From the SILAC experiment, 620 protein hits could not be matched back to the transcriptome data set. Since the majority of these unmatched protein hits were detected with BAQ values comparable to those of proteins that could be matched to mRNA counterparts (Figure 4, green box plot), it is rather unlikely that corresponding mRNAs of these hits have too low intensities so that they would be missed by RNAseq. One alternative explanation is that the conversion of Uniprot entries to Flybase FBgn entries was not optimal and that therefore some mRNA-protein pairs were lost in the

conversion process. Also, the few data points with $\log_2$ FPKM <0 may have arisen from falsely matched pairs. At the same time 9,379 hits in the RNAseq data set could not be matched to proteins hits in the SILAC data set. The RNAseq hits that could not be matched (Figure 4, red box plot) are mostly hits with relatively low expression levels that would most likely give rise to undetectable protein levels in the screen used here. In an attempt to compare protein with mRNA abundances, protein intensities as that were directly based on the iBAQ values calculated by the MaxQuant software were directly compared to FPKM values determined by RNAseq and plotted (Figure 4). Although the trend is far from linear (R^2=0.366), it is similar to values previously reported in the literature (Schwanhüusser et al. 2011). Also, the plot reveals that, in general, higher FPKM values are associated with higher protein intensities. The small cluster in the upper right corner with extremely high mRNA levels represents ribosomal proteins.

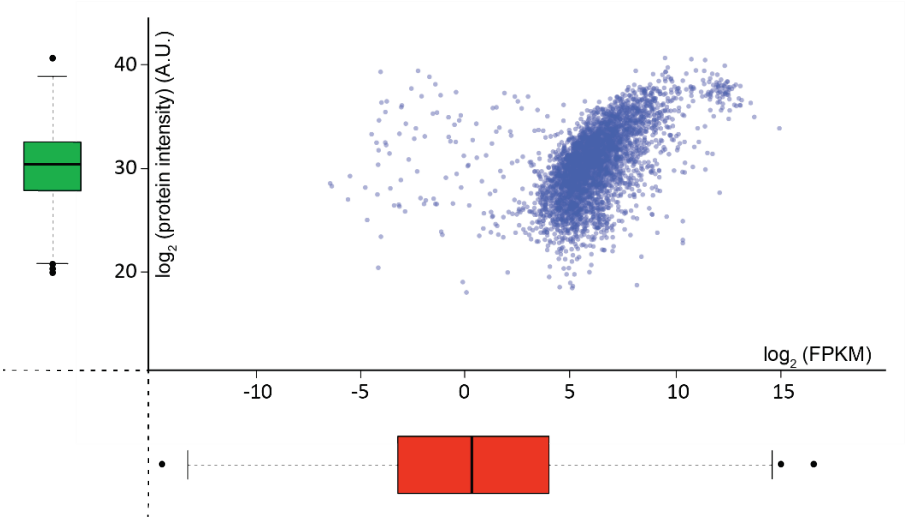

Figure 4. A scatter plot of absolute protein intensities (based on iBAQ values) versus absolute mRNA intensities (based on FPKM values) in untreated, steady state cells shows a relatively weak correlation (R^2=0.366). The intensity distribution of proteins for which no corresponding hit in the transcriptome analysis was found is represented by the green box plot. This distribution is very similar to the distribution of overlapping hits (blue data points). However, the distribution of mRNA hits for which no corresponding protein hit was identified (red box plot) shows that their average intensities are much lower than for overlapping hits. For these mRNA hits no corresponding protein hits were identified, most likely because protein concentrations were below the detection limit of the SILAC MS screen. The cluster of hits in the upper right corner with very high FPKM values represents mainly ribosomal proteins.

Clustering analysis revealed that the subset of proteins that are affected by ecdysone triggering could be clustered into 22 groups with similar trends in up- or downregulatory behavior (see Suppl Figure 2A). Several clusters show clear up- and downregulatory

effects and other clusters show interesting timing effects (*e.g.*, clusters 2 and 12). Most of the targets at the proteome level show large abundance changes only after longer incubation times and may therefore be the proteome analogs of late puff effects. Also, mRNA data were subjected to clustering analysis, indicating that many of the clusters are affected already after short incubation times. Some of the clusters show clear timing effects, for instance cluster 6, representing mRNAs that are downregulated at an early stage and then later stabilized.

However, gene ontology (GO) analysis on these clusters did not result in clear patterns of functional protein groups, so we decided to combine multiple clusters into higher-level clusters of up- and downregulated proteins (for complete data set see Suppl Table 3). Proteins that are downregulated upon ecdysone treatment include targets involved in nucleotide and carboxylic acid binding, as well as proteins involved in drug and RNA metabolism. Importantly, this group is enriched for proteins involved in post-embryonic development, for instance transcriptional regulators. The cluster of upregulated proteins is enriched for factors involved in stress response, aging and determination of adult life span. In addition, this set also includes proteins involved in post-embryonic development and morphogenesis, *e.g.* of neurons, and proteins involved in metabolism, catabolic processes and glutathione transferase activity. Examples of proteins involved in oxidation-reduction reactions, besides the known ecdysone response factor methionine sulfoxide reductase (Eip71CD, the *Drosophila* homolog of MsrA), are Cytochrome P450-4e2, Cytochrome P450-6a8 and Cytochrome P450-9f2. Because of their catalytic activity, these proteins are believed to be involved in the metabolism of insect hormones and in the breakdown of synthetic insecticides (Chen and Li 2007). Eip71CD, a repair enzyme for inactivated proteins by oxidation, is a downstream effector of FOXO signaling, which enhances resistance to oxidative stress and as such is believed to increase survival under stress conditions and extend lifespan (Chung et al. 2010) and is involved in neuron projection morphogenesis. Eip71CD is strongly upregulated, but protein levels decrease after very long incubation times. Other proteins that are involved in the response to oxidative stress include CG5346, which belongs to the iron/ascorbate-dependent oxidoreductase family, and CG6770. Interestingly, CG6770, a target gene of the putative FoxA protein Fork head (FKH) transcription factor and known to be induced by rapamycin, was found to be upregulated in the SILAC screen as a result of ecdysone triggering. FKH, besides its established role in embryonic development, controls cell and organismal size and is necessary for the expression of rapamycin and starvation responsive genes as well as for rapamycin induced inhibition of growth (Bülow et al. 2010). Knockdown of CG6770 (and of cabut) leads to increased cell size (Björklund et al. 2006), raising the possibility that it also acts as a negative regulator of growth downstream of FKH. It has been hypothesized that under conditions of dietary protein abundance, the Target Of Rapamycin (TOR) signaling module is active and exerts a negative regulation on FKH, which is

consequently sequestered in the cytoplasm and unable to modulate transcription of CG6770, cabut and Thor. However, when TOR complex 1 activity is inhibited by rapamycin or protein deprivation the repression of FKH activity is diminished, resulting in a significant fraction of the cellular FKH pool accumulating in the nucleus and then activates expression of the growth-inhibiting genes CG6770, cabut and Thor (Bülow et al. 2010). Although Thor was not identified in the SILAC screen, it was found to be upregulated in the RNAseq screen after 24 h ecdysone treatment, while, remarkably, cabut was slightly downregulated. LKR, a cofactor involved in the lysine ketoglutarate reductase / saccharopine dehydrogenase (SDH) process and involved in ecdysone-mediated transcription, was slightly upregulated.

When protein and mRNA abundance changes are directly compared, the delay effect that was observed at the proteome level is immediately clear at the 4 h time point (Figure 5A). Many of the RNAseq hits that are affected at an early stage do not show changes for their corresponding protein counterpart yet. After longer ecdysone treatments though, protein counterparts do show increased up- and downregulation (Figure 5B). In general, mRNA hits that are affected show changes in the same direction as their corresponding protein products (Figure 5B (red data points), Figure 5C), although in most cases the extent of mRNA up- or downregulation is one or two orders of magnitude higher. However, at longer time points several hits are down- or upregulated at the mRNA level that do not show a similar behavior at the proteome level (blue data points). For example, FTZ-F1, a nuclear hormone receptor that represses its own transcription and is repressed by ecdysone, was strongly downregulated at the transcriptome level, but remarkably did show no abundance changes at the proteome level after long ecdysone induction. This protein plays a central role in the prepupal genetic response to ecdysone and provides a molecular mechanism for stage-specific responses to steroid hormones (Woodard, Baehrecke, and Thummel 1994). Also, there are several hits that show an effect at the proteome level, but not at the transcriptome level (orange data points). The heatmap in Figure 5C illustrates that although upregulation of mRNA usually goes together with protein upregulation, mRNA downregulation not always results in lower protein levels, not even at the longer timescale. Finally, a small subset of hits reveals a mild antagonistic effect between mRNA and protein abundance changes, *i.e.* while protein expression goes up, mRNA expression goes down and *vice versa* (green data points). GO analysis reveals that group of proteins is enriched in functional categories such as post-embryonic development and nucleotide binding (see Suppl Table 3). Abundance fold changes for a selection of these proteins, as well as of proteins with clear timing effects, are plotted in Figure 5D. This set of proteins is, among others, enriched for post-embryonic development regulators and DNA binding proteins. Ken is a transcription factor required for terminalia development and is a negative regulator of the JAK/STAT pathway (Arbouzova, Bach, and Zeidler 2006; Lukacsovich et al. 2003). In our screen, Ken is downregulated at the mRNA level

and protein levels are decreased at intermediate time points, but the protein seems to stabilize at later stages. Regeneration (Rgn) is a developmental protein and is expressed in blastema cells during the regeneration of imaginal disks and is important for transdetermination, the switching of specific stem-cell like cells to a different fate (McClure, Sustar, and Schubiger 2008). Here, Rgn shows strong upregulation both at the mRNA and protein levels after short incubation times, but is then downregulated at later time points. Mus309 is downregulated at the mRNA levels and, although its protein product abundance is increased at early time points, it seems to be degraded at a later stage. MET (CG30344) is a multidrug efflux transporter and involved in excretion of toxins via renal tubules. It has been shown that exposure to methotrexate in the diet results in an increased expression of MET (Chahine and O'Donnell 2010; Chahine, Seabrooke, and O'Donnell 2012). It is first downregulated and subsequently upregulated; the protein levels show a similar trend as the mRNA levels, albeit with a delayed effect. Interestingly, the peptidases CG30046 and CG33713 are upregulated at an early stage, but later downregulated, whereas the peptidase CG17337 shows an opposite regulative trend. Proteins of uncharacterized function include CG15820 and CG15390. CG15820 is upregulated then downregulated and the protein abundance follows this trend. CG15390 is downregulated at the mRNA level, but at short and intermediate time points it is slightly upregulated at the protein level. Trol is downregulated at early time points, but strongly upregulated at longer time points. The protein Eve (Even skipped), which functions in the trol pathway, has been described to be rescued by the hormone ecdysone (Y. Park et al. 2001). In contrast, the oxidoreductase protein CG5346 is first upregulated and downregulated at later time points.

We speculate that in cases where abundance fold changes between the transcriptome and proteome levels do not correspond, there could be a more complex interplay between these different cellular acting levels. For instance, very different turnover rates of proteins and mRNAs may play a role, as well as posttranslational modifications (PTMs) on proteins that can affect their activity dramatically, but do not *per se* result in abundance changes at the global proteome level. Both turnover rate analysis and the characterization of PTMs were not taken into account in this study and are subject for further analysis. Overall, we observed a substantial overlap in terms of affected targets between the dynamic proteome and transcriptome after ecdysteroid induction. However, there are clear differences in timing effects between the transcriptome and proteome levels; effects in the proteome are usually delayed with respect to the changes in mRNA levels. Also, downregulation of mRNAs in many cases does not correlate to downregulation at the proteome level and in some cases there seems to be no correlation between transcriptome and proteome dynamics at all. Finally, we have found several proteome targets and players in the ecdysone signaling pathway that have not been described before.

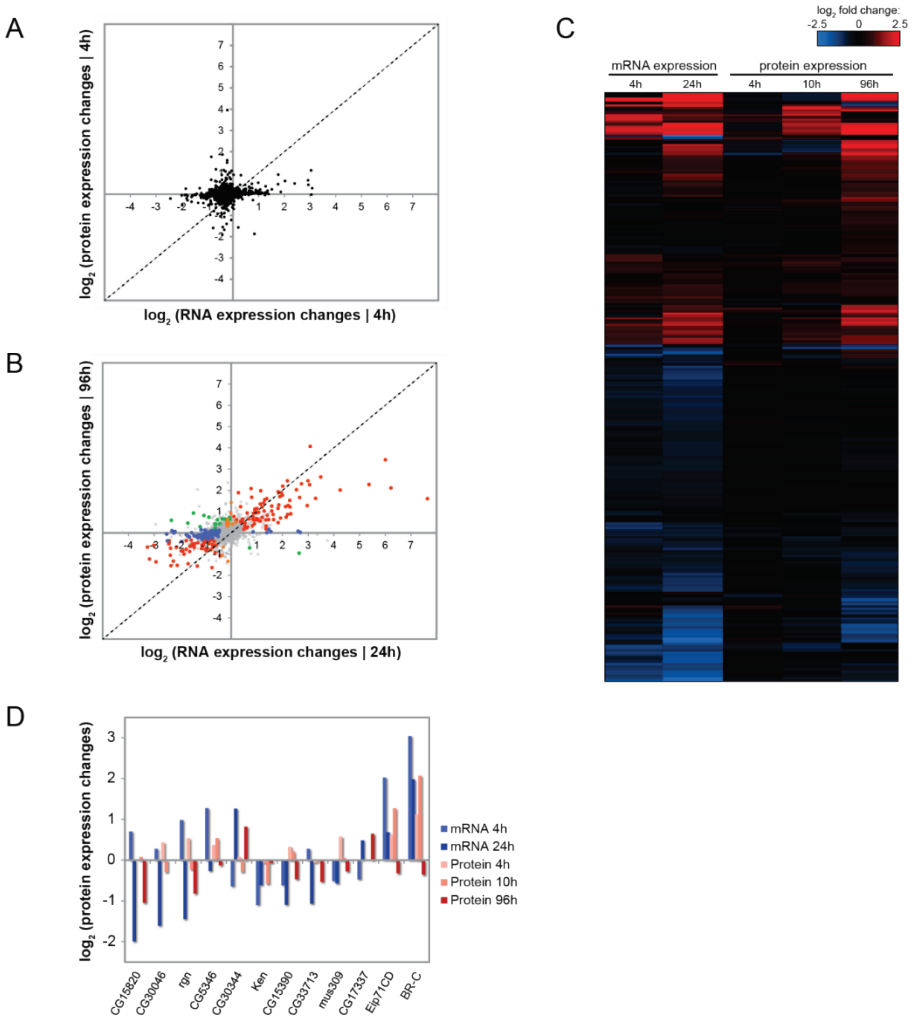

Figure 5. Relationship between mRNA and protein expression values for corresponding hits. **A)** Responses after short time points indicate that the early cellular products are mainly mRNAs, while the corresponding protein products are not (yet) up- or downregulated. **B)** Responses after longer incubation times reveal that hits can be grouped into clusters of specific dynamic behavior (see main text for details). **C)** Dynamics of a selection of overlapping targets from both transcriptome and proteome expression analyses. The majority of hits show a correlation between transcription and protein expression. However, especially for hits that are downregulated at the mRNA levels, in many cases the corresponding protein levels do not show a downregulation, not even after notably longer incubation times. **D)** Several hits show no correlation between mRNA and protein expression, or a remarkable timing effect.

Identification of EcR interaction partners

The ecdysone receptor is a type II nuclear receptor and is most commonly comprised of an EcR protein dimerized with USP. Upon ecdysteroid binding, the receptor activates ecdysone-responsive genes. Different isoforms of the EcR protein exist (Koelle et al. 1991) and various dimerization partners have been described (Bitra and Palli 2009), which supposedly allows the receptor to bind to different EcREs. Different genes can thus be activated and, as a result, the response to ecdysone can be specifically modulated. Here we set out to identify novel interaction partners for the EcR protein. Endogenous EcR was purified from *Drosophila* embryo nuclear extracts using two different commercial antibodies as the bait, Ag10.2 and DDA2.7 (DSHB), which both recognize a common epitope present in all three EcR isoforms (Ag10.2: amino acid residues 649-878, including the Gln/Pro-rich domain; DDA2.7: amino acid residues 335-393). Figure 6 highlights proteins identified in at least one of the IPs that were highly enriched compared to mock IP samples with a pre-immune-derived antibody mixture (for the complete data set see Suppl Table 5). Relative protein abundance values were based on peptide counts and the label free quantitation (LFQ) algorithm available in MaxQuant (Cox et al. 2014). Proteins were defined as being enriched when the LFQ intensity ratio between case and control samples was >3 and the MS/MS count ratio >4. Also, individual proteins were only included when the MS/MS count was at least 10, to obtain sufficiently reliable quantitation values. It should be noted though that the majority of hits have much higher LFQ intensity and MS/MS count ratios.

EcR was identified in both IPs, as well as its heterodimerization partner USP. Since EcR is a DNA binding nuclear receptor that can activate ecdysone-responsive genes in an RNA Pol II dependent manner, it is no surprise that several of the co-purified proteins and potential interaction partners play diverse roles in transcription. TFIIFalpha and TFIIFbeta, highly enriched in the Ag10.2 IP, are subunits of TFIIF, one of the general transcription factors that make up the RNA Pol II preinitiation complex. In addition, almost the complete Mediator complex, a coactivator of RNA Pol II dependent genes, was identified in the Ag10.2 screen. Other transcription related proteins include Mip120 (Lin-54 homolog), which negatively regulates transcription from the RNA Pol II promoter (Beall et al. 2007), the RNA Pol associated protein RTF1, CG3815 (Pf1), which is part of the Lid complex (Lee et al. 2009) and acts as a positive transcription regulator, and PCAF, part of the SAGA complex and involved in transcriptional regulation. Proteins involved in transcription that have been in one way or another linked to ecdysone signaling include transcription factor HNF-4 homolog (HNF4), a nuclear receptor with basal transcriptional activity that is a key regulator of lipid mobilization and β-oxidation in response to nutrient deprivation (Palanker et al. 2009). Another putative EcR interactor involved in transcriptional regulation is Domino, a SWI/SNF-like ATP-dependent chromatin remodeling enzyme that has been implicated in Notch signaling, as well as Nipped A (Gause et al. 2006).

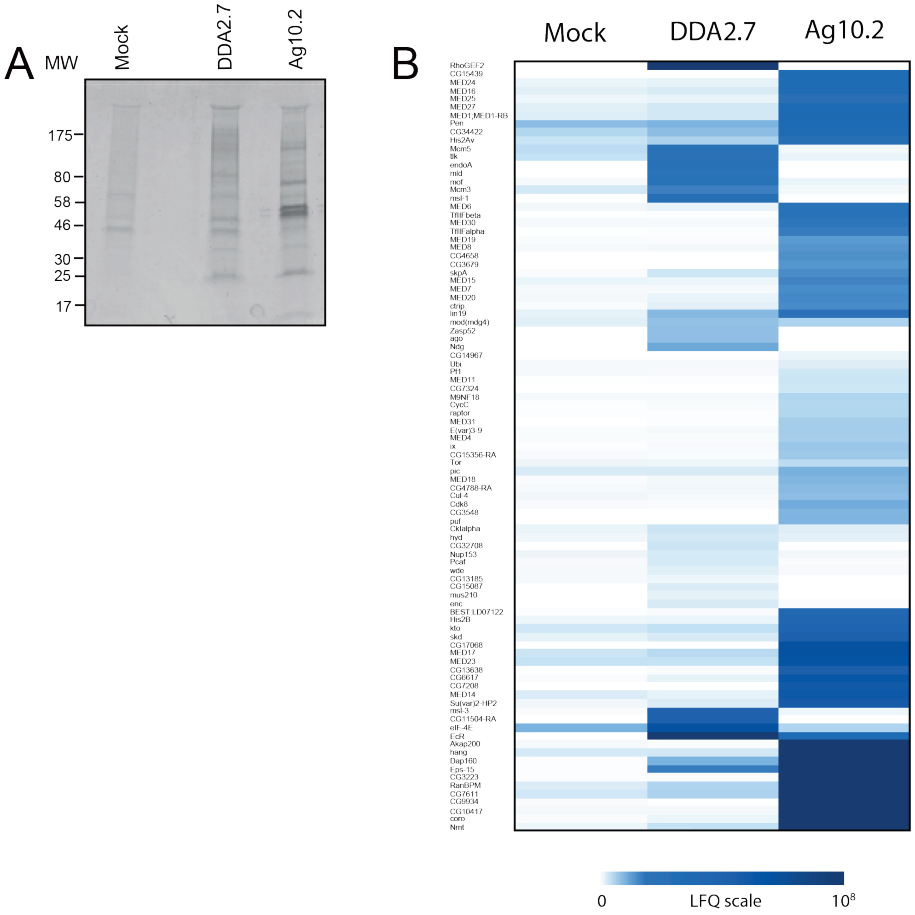

Figure 6 | For EcR IPs, DDA2.7 or Ag10.2 antibodies were first coupled to ProtG beads. Subsequently, IPs were performed in 1 ml Drosophila nuclear extract. The mock IP was performed using non-specific pre-immune serum coupled to ProtG beads. Beads were washed under relatively mild conditions and proteins were eluted and separated by SDS-PAGE. The gel was stained with Coomassie (A) and lanes were cut into slices and subjected to in-gel tryptic digestion. Peptides were identified by LC-MS/MS and analyzed by MaxQuant using the using label free quantitation (LFQ) module. Relevant hits are shown in the heatmap (B) where the color scale represents the range of LFQ values. Requirements of specific interactors for inclusion in the heatmap were: LFQ intensity case: control >3; MS/MS count ratio case: control >4; MS/MS count for individual proteins >10 and LFQ intensity >500,000. It should be noted though that the majority of hits have much higher LFQ intensity and MS/MS count ratios.

The histone methyltransferase Hmt4-20 that specifically trimethylates H4 K20 and therefore represents a specific tag for epigenetic transcriptional repression was enriched in the EcR IPs. Interestingly, trithorax-related (TRR), which did not show enriched LFQ values in this screen, specifically trimethylates H3 K4 and was previously identified as a coactivator for the ecdysone receptor (Sedkov et al. 2003). It is recruited by EcR in an ecdysone dependent manner and modulates chromatin at ecdysone inducible promoters. Another chromatin modifier that was identified is the histone acetyl transferase MOF, a component of the multisubunit histone acetyltransferase complex (MSL), which is - at least - composed of MOF, MSL1, MSL2 and MSL3. It is also part of a second histone acetyltransferase complex, the NSL complex, at least composed of MOF, NSL1/wah, NSL2/dgt1, NSL3/Rcd1, MCRS2/Rcd5, MBD-R2 and wds. MOF positively regulates sequence specific DNA binding transcription factor activity and is involved in dosage compensation (Gorman, Franke, and Baker 1995). MOF's interactors MSL1 and MSL3, but not MSL2, came down with EcR in our screen. Also, the chromatin modifying enzymes E(var)3-9, which has been implicated in an essential process during embryogenesis (Weiler 2007), Su(var)2-HP2 and the chromatin insulator Mod(mdg)4, a regulatory element that establishes independent domains of transcriptional activity within eukaryotic genomes, were specific for the EcR IP. Several subunits of the SCF (SKP1-CUL1-F-Box protein) E3 ubiquitin-protein ligase complex were identified: FBXW7 (ago), which mediates the ubiquitination and subsequent proteasomal degradation of target proteins, the cullin homolog lin19 and SkpA (Dredd) (Bader, Arama, and Steller 2010), as well as another F-box protein, FBX011, which has been described as a SkpA interacting protein (Stanyon et al. 2004). Encore (enc), specific for the DDA2.7 co-IP, has been shown to interact with Cyclin E, Cul1 (lin19), and components of the SCF-proteasome system (Ohlmeyer 2003). Several co-purified proteins play a role in fly development, such as in embryogenesis and oogenesis. The Ran binding protein homolog RanBPM, for instance, is involved in JAK/STAT signaling (Dansereau and Lasko 2008). Gustavus (gus) is a ubiquitin Cullin-RING E3 ligase expressed in nurse cells and is important for the polarity of the developing oocyte (Kugler et al. 2010). G protein G alpha i plays a role in glial cell differentiation during embryogenesis (Granderath et al. 1999). We also identified protein kinase A (PKA) regulatory and catalytic subunits, Pka-R2 and Pka-C1, respectively, which transduce signals through phosphorylation of different target proteins. Pka-R2 may play an essential role in the regulation of neuronal activity in the brain (S. K. Park et al. 2000). The zinc finger protein MLD is required for ecdysone biosynthesis and has been linked to different cellular processes, such as determination of adult lifespan, long-term memory development, and the regulation of the circadian sleep/wake cycle (Neubueser et al. 2005). Interestingly, two proteins involved in TOR signaling, raptor and target of rapamycin (TOR) (Wullschleger, Loewith, and Hall 2006) were co-purified with EcR. TOR regulates growth during animal development by coupling growth factor signaling to nutrient availability (Neufeld 2004). It is of notice that CG6770, which also plays a

role in TOR signaling, was previously identified in our SILAC screen as being strongly upregulated as a result of ecdysone induction. Altogether, these results may suggest a direct connection between ecdysone signaling and the TOR signaling pathway that regulates growth and needs further investigation. Two proteins, Hangover (Scholz 2005) and Akap200 (Kong 2010), are expressed ubiquitously in the nervous system, in neurons but not glia, and are required for normal development of ethanol tolerance. Several proteins involved in vesicle transport and phagocytosis were identified. Coronin (coro) is an actin binding protein which also interacts with microtubules and in some cell types is associated with phagocytosis (Bharathi 2004). Zizimin-related (Zir), a Rho guanine nucleotide exchange factor (RhoGEF) and homologous to the mammalian Dock-C/Zizimin-related family, plays a role in phagocytosis and is also important for the immune response in *Drosophila* (Sampson et al. 2012). Eps15 and its major binding partner Dap160 were both co-purified and control synaptic vesicle membrane retrieval and synapse development (Koh et al. 2007), but also have been suggested to negatively regulate Notch signaling (Tang et al. 2005). Furthermore, N-myristoyltransferase (NMT) is part of a family of myristoyl proteins, which are components of cellular signaling pathways and play important roles during embryonic development, making NMT essential for embryogenesis (Ntwasa 1997). Finally, several proteins involved in the ubiquitin-proteasome system and neurogenesis (Ctrip, CG42574) and DNA repair and replication (*e.g.*, hay and MCM3) were identified as putative interactors. Several yet uncharacterized proteins were found to be specific for the EcR IP, such as CG34422, CG7611 (WDR26), CG6617 and CG13638; these require further analysis.

Although several proteins were identified with high scores and - presumably – in relative high abundances as compared to the mock IP, and therefore seem to be *bona fide* interactor candidates, the overlap between two IPs with different antibody is only rather poor. The reason for this is unknown, although it can be speculated that steric inhibition of EcR antibody binding sites may mask different interaction domains, since the antibodies were raised using different antigen epitopes. Alternative strategies to clarify this would be the construction of tagged versions of the receptor for affinity purification or raising antibodies using partially non-overlapping epitopes. Also, it would be of great interest to investigate the dynamics with quantitative proteomic techniques of EcR interactions and of the EcR/USP dimer in the presence of ecdysone, which introduces a conformational change leading to transcriptional activation of genes under the EcRE control.

Conclusive remarks

In conclusion, we have performed a protein-protein interaction analysis of EcR, a target protein of ecdysone, to shed more light on ecdysone signaling at the interactome and proteome level. Proteins co-purified with EcR include factors involved in RNA Pol II dependent transcription and chromatin modifying enzymes. Also, several proteins previously linked to ecdysone signaling and/or biosynthesis were identified. The identification of several proteins linked to both the TOR and Notch signaling pathways could potentially be interesting targets for follow-up studies and might offer new insights in ecdysone induced protein expression. In addition, we have performed a quantitative, proteome-wide screen to monitor the effect of ecdysone administration to *Drosophila* Kc cells. We have compared changes at the proteome level to more upstream effects at the transcriptome level. We observed a substantial overlap in terms of affected targets between the dynamic proteome and transcriptome after ecdysteroid induction. However, there are clear differences in timing effects between the transcriptome and proteome levels; effects in the proteome are usually delayed with respect to the changes in the transcriptome. Also, downregulation of mRNAs in many cases does not correlate to downregulation at the proteome level and in some cases there seems to be no correlation between transcriptome and proteome dynamics whatsoever. Finally, we have found several proteome targets and players in the ecdysone signaling pathway that have not been described before. It would be of great interest to extrapolate these quantitative proteomics studies from cultured cells to the fly, which is technically possible (Sury, Chen, and Selbach 2010, Gouw et al. 2011) and would allow for the investigation of ecdysone action in a developmental stage dependent and/or even in an organ specific manner.

Experimental methods

20-Hydroxyecdysone treatment. *Drosophila melanogaster* Kc cells were treated with 1 μM 20-hydroxyecdysone (H5142, Sigma) dissolved in DMSO, or mock-treated with an equal volume of DMSO for 4, 10, 16, 24, 48 or 96 h.

Antibodies and immunoblotting. Cells were washed 3x with cold PBS and lysed in 2x Laemmli sample buffer. Samples were sonicated using a Bioruptor (Diagenode) for 5 min, 30 seconds 'on' and 30 seconds 'off' cycles and boiled at 95 °C. Proteins were resolved by SDS-PAGE and blotted to PVDF membranes. Membranes were blocked with 10% dry milk in PBST (PBS with 0.1% Tween), incubated with primary antibodies in PBS/3% BSA. Following multiple PBST washes, membranes were incubated with alkaline phosphatase (AP) secondary antibodies. Membranes were washed with PBST and developed with NBT/BCIP.

Antibodies. Antibodies against Broad-Complex (BR-C) (α-Broad-core, 25E9.D7) and EcR (DDA2.7 and Ag10.2) were from the Developmental Studies Hybridoma Bank (DSHB); antibodies against H2B (α-H2B, 07-371) were from Millipore.

SILAC and sample preparation for global proteome analysis. Kc cells were cultured in custom made Schneider's *Drosophila* medium (Athena Enzyme Systems, Baltimore, MD), based on Invitrogen's formulation (Invitrogen, #21720-001), with following modifications: dialyzed yeastolate (3500 Da MWCO) and deficient for lysine and arginine. Before use, the medium was supplemented with 5% dialyzed fetal bovine serum (F0392, Sigma-Aldrich), 1% penicillin-streptomycin and 2 mg/ml 'light' ($^{12}C_6$) lysine (A6969, Sigma-Aldrich) and 0.5 mg/ml 'light' ($^{12}C_6$ and $^{14}N_4$) arginine (L5751, Sigma-Aldrich), or 'heavy' ($^{13}C_6$) lysine (CLM-2247, Cambridge Isotope laboratories) and 'heavy' ($^{13}C_6$ and $^{15}N_4$) arginine (CNLM-539, Cambridge Isotope Laboratories). Cells were cultured at 27 °C for at least 7 cell doublings to reach complete labeling. Experiments were done in a forward and reverse manner: in forward, light cells were treated with ecdysone and heavy cells were treated with DMSO, and *vice versa* in the reverse experiment. After ecdysone treatment, $30x10^6$ heavy labeled and $30x10^6$ light labeled cells were mixed. Cells were washed with cold PBS (3x) and lysed in 100µl 2x Laemmli sample buffer. Samples were sonicated using a Bioruptor (Diagenode) for 5 min, with 30 seconds 'on' and 30 seconds 'off' cycles and boiled at 95 °C. For the LTQ-Orbitrap workflow (see LC-MS/MS section), proteins were resolved on a 12% SDS-PAGE gel and visualized by Coomassie staining. Lanes were cut in 1mm slices and combined to 80 fractions per lane and analyzed by LC-MS/MS. Alternatively, for the Q Exactive workflow, proteins extracts were digested and fractionated by HILIC on an Agilent 1100 HPLC system using a 5 µm particle size 4.6 x 250 mm TSKgel amide-80 column (Tosoh Biosciences). 200 µg of the desalted tryptic digest was loaded onto the column in 80% acetonitrile. Next, peptides were eluted using a nonlinear gradient from 80% B (100 % acetonitrile) to 100% A (20 mM ammonium formate in water) with a flow of 1 ml/min. Sixteen 6 ml fractions were collected, lyophilized and pooled into 8 final fractions. Each fraction was then analyzed by LC-MS/MS.

Immunopurification. Nuclear extracts (NE) from 0-12 h old *Drosophila* embryos were prepared as described (Chalkley and Verrijzer 2004). Immunopurification (IP) procedures were performed essentially as described (Chalkley and Verrijzer 2004). Briefly, DDA2.7 antibody (1.5 ml, 142 µg/ml) was crosslinked to 100 µl ProtG beads (GE Healthcare) and Ag10.2 antibody (0.5 ml, 207 µg/ml) was crosslinked to 50 µl ProtG beads by using dimethylpimelimidate. As a control, antibodies from pre-immune serum were coupled to ProtG beads. After 2 h incubation of the antibody coupled beads with NE, the beads were washed extensively with HEMG buffer (25 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 200 mM KCl, 0.1% NP-40, containing a cocktail of protease inhibitors). Proteins retained on the beads were eluted with 100 mM sodium citrate buffer (pH 2.5), resolved by SDS-PAGE and

visualized by Coomassie staining. Lanes were cut in 1 mm slices and combined to in total 12 fractions per lane and analyzed by LC-MS/MS.

LC-MS/MS. In-gel protein reduction, alkylation and tryptic digestion was done as described previously (van den Berg et al. 2010). Peptides were extracted with 30% acetonitrile 0.5% formic acid and analyzed on an 1100 series capillary LC system (Agilent Technologies) coupled to a LTQ-Orbitrap hybrid mass spectrometer (Thermo) or on an EASY-nLC system (Thermo) coupled to a Q Exactive mass spectrometer (Thermo). Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 2 cm × 100 μm, packed in-house) at a flow rate of 8 μl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 75 μm, packed in-house) using a linear gradient from 0 to 50% B (A = 0.1% formic acid; B = 80% (v/v) acetonitrile, 0.1% formic acid) in 120 min (for IP samples) or 180 min (for SILAC global proteome samples) and at a constant flow rate of 300 nl/min (using a splitter for the 1100 system). The column eluent was directly electrosprayed into the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent acquisition mode by CID using top 8 selection (LTQ-Orbitrap) or HCD using top 15 selection (Q Exactive). Additional settings for Q Exactive operation: MS resolution: 70,000; MS AGC target 3E6; MS maximum injection time: 100 ms; MS scan range 375-1400 m/z; MS/MS resolution: 17,500; MS/MS AGC target: 1E5; MS/MS maximum injection time: 200 ms; intensity threshold: 5E3.

Mass spectrometry data analysis. RAW files were analyzed using MaxQuant software (v1.3.0.5 | http://www.maxquant.org), which includes the Andromeda search algorithm (Cox et al. 2011) for searching against the Uniprot database (version December 2013, taxonomy: *Drosophila melanogaster* | http://www.uniprot.org/). Follow-up data analysis was performed using the Perseus analysis framework (http://www.perseus-framework.org/), the GProX proteomics data analysis software package (v1.1.12, http://gprox.sourceforge.net/) (Rigbolt, Vanselow, and Blagoev 2011) or in-house developed software. The 'Significance B' option in the Perseus software suite (Cox and Mann 2008), which takes into account the intensity of peptides/proteins, was used to determine significant outliers to determine significant outliers (p-value < 0.05). Additionally, only proteins that were up- or downregulated by at least 1.5-fold were selected for follow-up analysis. An extra requirement to maintain a high quality data set was the presence of consistent ratios in forward and reverse experiments, so a protein hit with a SILAC ratio of >1.5 ($\log_2$ ratio >0.585) in forward should also have a SILAC ratio of <0.66 ($\log_2$ ratio <-0.585) in the reverse experiment and *vice versa*. Relative protein intensities within a sample were directly inferred from the iBAQ values in the label free quantitation module in MaxQuant (Schwanhüusser et al. 2011; Cox et al. 2014).

RNA isolation, quantitative real-time RT-PCR, RNA sequencing and data analysis. Total RNA was extracted from $5x10^6$ cells using Trizol (15596-026, Invitrogen) and 4 µg RNA was used for random hexamer primed cDNA synthesis using the Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed on a CFX96 realtime PCR detection system (Bio-Rad). Reactions were performed in a total volume of 25 µl containing 1x reaction buffer, SYBR Green I (Sigma), 200 µM dNTPs, 1.5 mM $MgCl_2$, platinum Taq polymerase (Invitrogen), 500 nM of corresponding primers and 1 µl of cDNA. The primer sequences used were: CG11874: 5'-AGTGTTGCTCTGCCTAAGTGG-3', 5'-CGGATGATGGTGCGGATTGG-3'; E75A: 5'-CCTTTCATTGACTAACTGCCACTC-3', 5'-CGAAACGAAACGAACGGAACG-3'; E23: 5'-CATCACGAGTAGCCACCATAAC-3', 5'-GGTTGGAGCGTTGATTGTAATAG -3'. Data analysis was performed by applying the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Values obtained from amplification of alpha-mannosidase-Ib (CG11874) were used to normalize the data as described previously (Moshkin et al. 2007; van der Knaap et al. 2010). Average amplification of three replicates is shown in graphs. Total RNA was purified from $5x10^6$ cells per time point according to Trizol protocol (15596-026, Invitrogen). An indexed sequencing library was obtained from total RNA with the Illumina TruSeq RNA (v4) kit. The libraries were pooled together, and sequenced on two lanes of a flow cell on a Illumina Hiseq 2000 and sequenced for 36 bp + 7 bp index using Illumina v3 chemistry. Illumina BaseCall results were demultiplexed using NARWHAL (Brouwer et al. 2012). The reads were aligned using Tophat (version 1.3.1, (Trapnell, Pachter, and Salzberg 2009)) against the UCSC dm3 reference genome, using Ensembl genes.gtf annotation provided by Illumina iGenomes (http://www.illumina.com/, downloaded February 2013). FPKM (Fragments Per Kilobase transcript per Million mapped reads) expression levels were calculated by Cufflinks (version 1.0.3, (Trapnell et al. 2010)). Differential gene expression output was generated with Cuffdiff using fragment length of 300 bases. Differential gene expression data was integrated with differential protein expression data using in-house developed software. Briefly, comparisons for the RNAseq datasets 'mock-4h', 'mock-24h' and '4h-24h' each yielded a gene_exp.diff file and these were loaded into PostgreSQL (http://www.postgresql.org/) database tables. Each has a column 'gene_id' that contains the gene name. Next, SILAC data in the format of regular MaxQuant output files were also loaded as PostgreSQL database tables. Since the two data sources (SILAC versus RNAseq) did not use the same identifier system (MaxQuant uses Uniprot accession numbers, while RNAseq data contain the gene names from the Illumina file), accession - gene name mapping tables were generated from the full Uniprot proteome text records. As expected, virtually all RNAseq names could be mapped onto Uniprot accessions. Via this mapping the two datasets were joined together to produce a table for direct comparisons.

Online supplementary information available:

Supplementary Figure 1. Quantitative real-time RT-PCR reveals that the mRNA products of the genes E23 and E75A, which have been described previously to be early responsive genes upon ecdysone treatment, are substantially upregulated in *Drosophila* Kc cells after hormone treatment.

Supplementary Figure 2. A) Clustering analysis shows that, although the majority of protein abundances do not change, the proteins that do show an effect can be divided into specific groups according their up- or downregulation behavior (GProX parameters for SILAC data: upper regulation threshold 0.5; lower regulation threshold -0.5; standardized: false; fuzzification value: 2; iterations: 100; expression changes are plotted on a $\log_2$ scale). **B)** Clustering analysis of mRNA abundance changes. Also here, although the majority of relative abundances of mRNAs do not change, the mRNAs that do change can be divided into specific groups according their up- or downregulation behavior (GProx parameters for RNAseqdata: upper regulation threshold 0.41; lower regulation threshold -0.41; standardized: false; fuzzification value: 2; iterations: 100; expression changes are plotted on a ln scale). Only RNA data are shown for which there are overlapping hits between SILAC and RNAseq assays.

Supplementary Figure 3. Pie charts summarizing the GO functional annotation analysis for downregulated, upregulated and non-correlated protein-mRNA pairs after stimulation of Kc cells with ecdysone.

Supplementary Table 1. SILAC cell culture growth medium formula for insect cells.

Supplementary Table 2. MaxQuant SILAC data and RNAseq data including the overlap between these two data sets.

Supplementary Table 3. GO enrichment analysis of hits that are upregulated or downregulated (>1.5 fold) at the mRNA and protein levels, and proteins that show non-correlated behavior compared to the corresponding mRNA levels after ecdysone treatment.

Supplementary Table 4. Expression changes at the mRNA and protein levels for the hits shown in heatmap Figure 5C.

Supplementary Table 5. MaxQuant EcR IP data.

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