Transcriptional Networks Controlling Adipocyte Differentiation

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Adipocyte differentiation is regulated by a complex cascade of signals that drive the transcriptional reprogramming of the fibroblastic precursors. Genome-wide analyses of chromatin accessibility and binding of adipogenic transcription factors make it possible to generate “snapshots” of the transcription factor networks operating at specific time points during adipogenesis. Using such global “snapshots,” we have demonstrated that dramatic remodeling of the chromatin template occurs within the first few hours following adipogenic stimulation and that many of the early transcription factors bind in a cooperative fashion to transcription factor hotspots. Such hotspots are likely to represent key chromatin nodes, where many adipogenic signaling pathways converge to drive the adipogenic transcriptional reprogramming.

INTRODUCTION

Models of Adipocyte Differentiation

Adipocytes develop from mesenchymal stem cells (MSCs) in adipose tissue and to a lesser extent in various other tissues. This process involves an initial commitment phase where the MSCs are committed to the adipocyte lineage, followed by a differentiation phase where the preadipocytes develop into mature fat-laden adipocytes. Although, relatively little is known about the signaling pathways controlling commitment, the differentiation of preadipocytes to adipocytes (adipogenesis) is one of the best studied differentiation processes. Results obtained by a large number of laboratories using gain- and loss-of-function approaches have identified multiple interconnected signaling pathways controlling this differentiation process (Hwang et al. 1997; Rosen and Spiegelman 2000; MacDougald and Mandrup 2002; Farmer 2006; Rosen and MacDougald 2006; Lefterova and Lazar 2009; Siersbæk et al. 2010). The majority of discoveries of important adipogenic signaling pathways have been based on investigations in cell lines derived from mouse embryonic fibroblasts (Hwang et al. 1997), in particular the 3T3-L1 cell line (Green and Kehinde 1975). Although all these cell lines require a pharmacological cocktail of inducers to initiate the differentiation process, subsequent *in vivo* experiments have convincingly validated the physiological importance of the majority of these signaling pathways (Valet et al. 2002; Rosen and MacDougald 2006). Thus, these *in vitro* differentiation models represent a valuable tool for the identification of novel signaling networks regulating adipogenesis.

External Signals Inducing Adipogenesis

The adipogenic conversion of most mouse and human preadipocyte cell lines, including the murine 3T3-L1 cell line, requires stimulation with a pharmacological cocktail (MDI; Hwang et al. 1997). This cocktail consists of the synthetic glucocorticoid dexamethasone, the cAMP elevating agent 1-methyl-3-isobutyl xanthine (MIX), and pharmacological doses of insulin, which activates both the insulin receptor (IR) and the insulin-like growth factor receptor (IGF-R; Green and Kehinde 1975; Student et al. 1980; Schmidt et al. 1990; Yeh et al. 1995). In addition, efficient differentiation of many primary fibroblastic precursors requires supplementation with high affinity agonists of the peroxisome proliferator activated receptor γ (PPAR γ ; Wabitsch et al. 2001; Hansen et al. 2002; Janderova et al. 2003; Petrovic et al. 2010). The supremacy of the 3T3-L1 cell culture model for the study of adipogenesis is due to the fact that these cells, unlike most other models, can be brought to undergo a rather synchronous and complete differentiation, which does not require pharmacological activation of PPAR γ . Thus, when the MDI cocktail in combination with fetal calf serum is administered to confluent 3T3-L1 cells, the cells undergo mitotic clonal expansion followed by growth arrest and induction of the adipocyte phenotype (Tang et al. 2003b). Lipid droplets become apparent 3–4 d after exposure to the cocktail.

The components of the MDI cocktail were originally empirically derived, and we have only recently begun to understand the downstream effector cascades. Dexamethasone is a key component of the adipogenic cocktail and is believed to induce the gene program by activating

the glucocorticoid receptor (GR; Yeh et al. 1995; Pantoja et al. 2008). Consistent with this, knockdown of GR interfered with MDI-induced differentiation of 3T3-L1 preadipocytes (Steger et al. 2010). However, the mineralocorticoid receptor (MR) can also bind dexamethasone, and this receptor may also mediate additional effects of glucocorticoids on adipocyte differentiation (Caprio et al. 2007, 2011).

MIX is a phosphodiesterase inhibitor and therefore leads to elevated levels of cAMP, which activate protein kinase A (PKA; Reusch et al. 2000), as well as members of the family of exchange proteins directly activated by cAMP (Epac; Petersen et al. 2008). The cAMP response element-binding protein (CREB) is known to be activated by PKA-mediated phosphorylation and was originally thought to be the main effector of the cAMP signal (Reusch et al. 2000). However, more recent data indicate that PKA is dispensable for the CREB phosphorylation by MDI, which appears to rely more on activation of extracellular signal-regulated kinase 1/2 (ERK1/2), whereas PKA plays an important role in inhibition of Rho and Rho kinase activity (Petersen et al. 2008). Thus, it is likely that the signaling cascade elicited by cAMP is much more complex than first anticipated and that several other transcription factors are directly or indirectly targeted by PKA and Epac. Potential candidates are CCAAT/enhancer-binding protein (C/EBP β ; Tang et al. 2005) and Krüppel-like factor 4 (KLF4; Birsoy et al. 2008).

Insulin is administered in pharmacological doses and thus also activates the IGF-R, through which it is believed to stimulate the early phase of adipogenesis (Smith et al. 1988; Boney et al. 1998). IGF-R activates the ERK 1/2 and the phosphoinositide 3-kinase (PI3K) pathways, both of which appear to be required for adipogenesis (Sale et al. 1995; Sakaue et al. 1998; Tang et al. 2005), although the role of ERK 1/2 is controversial (Font de Mora et al. 1997). Downstream effects of insulin/IGF-1 signaling include activation of C/EBP β (Tang et al. 2005), the sterol regulatory element-binding protein 1 (SREBP-1; Lay et al. 2002), and CREB (Petersen et al. 2008), as well as inhibition of forkhead box protein O1 (FOXO1; Fan et al. 2009; Gerin et al. 2009).

Finally, growth hormone (GH) from the fetal calf serum has been shown to activate signal transducer and activator of transcription 5a/b (Stat5a/b) through Janus kinase-2 (JAK-2) (Yarwood et al. 1999), as well as C/EBP β through activation of ERK 1/2 (Cui et al. 2011).

Transcriptional Cascades Regulating Adipogenesis

The transcriptional regulation of adipocyte differentiation is known to rely on an intricate interplay between multiple adipogenic transcription factors. Among these PPAR γ stands out as a key regulator obligate for *in vitro* as well as *in vivo* development of adipocytes and the only factor that can induce an adipocyte-like phenotype upon ectopic expression in non-adipogenic cell types (Tontonoz and Spiegelman 2008). However, the function of PPAR γ is tightly linked to members of the C/EBP

family, which have well-documented important functions at different time points during adipocyte differentiation. C/EBP β and - δ are already expressed in preadipocytes but are rapidly further induced (Cao et al. 1991; Tang and Lane 1999; Salma et al. 2006; Grøntved et al. 2010) and posttranslationally activated (Tang et al. 2005) by the adipogenic cocktail. The expression of C/EBP β is induced by CREB and GR, whereas C/EBP δ is induced by GR (Cao et al. 1991; Pantoja et al. 2008). In addition, the ERK 1/2 pathway induces phosphorylation and activation of C/EBP β (Tang et al. 2005). C/EBP β and - δ are direct activators of the expression of PPAR γ and C/EBP α (Hamm et al. 2001; Salma et al. 2004, 2006), the two major late-acting adipogenic transcription factors. These two transcription factors have been shown to mutually induce the expression of each other and to cooperate in the activation of the adipogenic gene program (Wu et al. 1999; Farmer 2006).

Although PPAR γ and members of the C/EBP family form the classical core transcriptional network of adipogenesis, multiple other transcription factors have been demonstrated to play an important role, either early in differentiation or at later stages. These factors are linked together in a causal and temporal network, which forms at least two separate waves of adipogenic transcription factors (Fig. 1). In addition to C/EBP β and - δ , the first wave consists of early activators such as GR (Steger et al. 2010), Stat5a/b (Floyd and Stephens 2003), CREB (Reusch et al. 2000), KLF4 (Birsoy et al. 2008), KLF5 (Oishi et al. 2005) and Krox20 (Chen et al. 2005). Factors of the early wave directly or indirectly impact on the expression of factors of the second wave. The transcription factors of this wave, in particular PPAR γ and C/EBP α , drive the final adipocyte differentiation and/or modulate adipocyte metabolism. Factors such as SREBP-1 (Kim and Spiegelman 1996) and KLF15 (Mori et al. 2005) have been shown to play a role in adipocyte differentiation. The liver X receptor (LXR) modulates lipogenesis (Ross et al. 2002) and regulates expression of SREBP-1 (Repa et al. 2000) and PPAR γ (Juvet et al. 2003).

GENOME-WIDE INVESTIGATIONS OF TRANSCRIPTIONAL NETWORKS REGULATING ADIPOGENESIS

Genome-Wide Technologies and Global Maps of Transcription Factor Networks

The recent advances in deep sequencing combined with chromatin immunoprecipitation (ChIP-seq) have enabled extensive mapping of transcription factors as well as epigenetic marks at a genome-wide level. We and others have used ChIP-seq as well as other global approaches to generate genome-wide binding profiles of PPAR γ and C/EBP α and other transcription factors and epigenetic marks during adipocyte differentiation of murine (Lefterova et al. 2008; Nielsen et al. 2008; Hamza et al. 2009; Wakabayashi et al. 2009; Mikkelsen et al. 2010; Steger et al. 2010; Yu et al. 2010; reviewed

Figure 1. Outline of the transcriptional cascade controlling adipocyte differentiation. The adipogenic cocktail of inducers (i.e., insulin, glucocorticoids, a cAMP elevating agent, and serum containing high levels of growth hormone) activates the first wave of adipogenic transcription factors, which jointly induce the second wave of factors. The second wave, in which PPAR γ and C/EBP α play a key role, directly induces and maintains the mature adipocyte phenotype. For simplicity, only a subset of transcription factors and interconnections are indicated.

in Siersbæk et al. 2010) and human adipocytes (Mikkelsen et al. 2010; Lo et al. 2011; Schmidt et al. 2011; Soccio et al. 2011). These genome-wide maps have greatly changed our view on transcriptional regulation of adipogenesis. First, the master regulator PPAR γ is associated with the majority of genes that are induced during adipogenesis, indicating that PPAR γ may be directly involved in the regulation of most genes linked to the adipocyte phenotype. Second, genes that are known to be regulated by PPAR γ usually display multiple binding sites of PPAR γ in the vicinity of the gene loci, and by far the majority of the identified binding sites are in intronic or intergenic regions. The same is the case for many other transcription factors. Thus, the sites most frequently investigated in reporter constructs, that is, sites near the proximal promoter, may only represent a fraction of the regulatory regime of a particular gene, and it may not even represent the most important regulatory site. Third, many transcription factors bind to the same regions as other transcription factors, indicating that there is significant cross talk between different transcription factors at the level of chromatin. This extensive sharing of binding sites/regions begs for investigations focusing on transcriptional networks in the sense of “snapshots” of global interconnections between transcription factors on chromatin. Such global “snapshots” focus on the functional interaction between transcription factors on chromatin at a given time, whereas the traditional temporal networks focus on when and how transcription factors are induced.

Chromatin Remodeling during Adipogenesis

The tight wrapping of DNA around histones to form nucleosomes restricts transcription factor access to target DNA elements embedded in chromatin. Thus, transcription factor binding directly to DNA is usually associated with remodeling of the chromatin structure to increase DNA accessibility. Because remodeling complexes are recruited to DNA by transcription factors,

DNA accessibility is both a cause and a consequence of transcription factor binding to DNA. Open remodeled regions can be detected by their hypersensitivity to attack by endonucleases such as DNase I and restriction enzymes. These DNase I hypersensitive (DHS) sites are likely to represent important “action points” in chromatin where transcription factors and other sequence specific proteins bind to DNA. Traditionally, individual DHS sites have been detected by Southern blotting (Wu et al. 1979; Wu 1980; Keene et al. 1981) and more recently by quantitative polymerase chain reaction (PCR; McArthur et al. 2001; Dorschner et al. 2004; Boyle et al. 2008; John et al. 2008). These techniques are well suited for quantification of chromatin accessibility at specific genomic regions; however, they are limited by their low throughput. To obtain a global view of the chromatin landscape in a given cell type, small DNA fragments released from limited DNase I digestions have recently been analyzed by microarray (DHS-chip; Crawford et al. 2006a; Sabo et al. 2006; Boyle et al. 2008) and deep sequencing (DHS-seq; Crawford et al. 2006b; Boyle et al. 2008; Hesselberth et al. 2009; Stitzel et al. 2010; John et al. 2011), resulting in the generation of genome-wide maps of cell type-specific putative regulatory elements.

We recently employed DHS-seq to generate genome-wide maps of chromatin accessibility and thereby important “action points” in chromatin at various stages of 3T3-L1 adipocyte differentiation, that is, at 0 h, 2 h, 4 h, 1 d, 2 d, and 6 d following stimulation with the adipogenic cocktail (Siersbæk et al. 2011). So far only very few studies have investigated how the global pattern of chromatin accessibility changes in response to external stimuli (Biddie et al. 2011; Hurtado et al. 2011; John et al. 2011) or during development (Li et al. 2011; Thomas et al. 2011). Thus, our study of chromatin remodeling during adipogenesis represents one of the first studies to investigate changes in overall chromatin structure at a genome-wide level during a biological transition, and to our knowledge, the first to investigate this during a mammalian differentiation process.

Figure 2. The four major modes of chromatin remodeling during 3T3-L1 adipocyte differentiation. Genomic regions that develop hypersensitivity to DNase I at some time point during 3T3-L1 adipogenesis were clustered based on their temporal profile of accessibility (i.e., sensitivity to DNase I). This resulted in four groups of genomic “action points” with distinct temporal profiles of accessibility (Siersbæk et al. 2011). TF, transcription factor.

We identified ~35,000 genomic regions that are DNase I hypersensitive at one or more of the investigated time points during adipogenesis (Siersbæk et al. 2011). Interestingly, by analyzing the development of DHS sites during differentiation, we detected a dramatic increase in the number of DHS sites already within 4 h after administration of the adipogenic hormone cocktail (Fig. 2). A subset of these early remodeled sites has a transiently open chromatin configuration that peaks at the 4 h time point (Fig. 2, profile 2), and these sites are enriched in the vicinity of early induced genes linked to translation, mitochondrion, and glucose catabolism. This suggests that these regulatory regions are involved in the initial reprogramming of transcription during differentiation. In contrast to these transiently open regions, a large proportion of the sites that are remodeled at 4 h maintain an open chromatin structure throughout the differentiation process (Fig. 2, profile 1). These early remodeled regions are enriched in the vicinity of both early and late induced genes, suggesting that they represent regulatory sites involved in transcriptional regulation of genes activated at both early and late stages of differentiation. This would imply that chromatin remodeling at some regulatory sites precedes transcriptional activation of late induced genes.

Another group of genomic regions peak in DNase I accessibility at day 1 of differentiation and display a closed chromatin configuration in mature adipocytes (Fig. 2, profile 3). These regions are enriched in the vicinity of genes that are transiently induced at day 1, suggesting that adipogenic transcription factors transiently associate with many of these regions to drive expression of neighboring genes. The gene program that is transiently activated at this stage of differentiation consists of genes linked to DNA packaging, translation, and cell cycle, consistent with the fact that the cells go through mitotic clonal expansion at this early stage prior to terminal differentiation.

Coincident with the activation of the second wave of adipogenic transcription factors, a final group of genomic regions are remodeled around day 2 of differentiation and the open chromatin configuration at these sites persists

throughout terminal differentiation (Fig. 2, profile 4). These late remodeled regions are highly enriched in the vicinity of genes induced late in adipogenesis, many of which are linked to lipid and glucose metabolism. This suggests that these regions are involved in the final adipogenic reprogramming of transcription that is important for acquisition of the mature adipocyte phenotype.

Hotspots in the Early Wave of Adipogenic Factors

Based on the dramatic remodeling of chromatin occurring within 4 h following addition of the adipogenic cocktail, we chose to focus our investigations on obtaining a “snapshot” of the transcriptional network established at the 4 h time point (Siersbæk et al. 2011). Using de novo motif search of the 6000 most prominent new DHS peaks at that time point, we identified enrichment of binding motifs for multiple factors of potential interest including GR, Stat5, and C/EBPs. ChIP-seq profiles of these factors as well as the retinoid X receptor, which we have previously documented to be binding to chromatin early in differentiation (Nielsen et al. 2008), demonstrated that binding of all factors is indeed enriched at DHS sites at 4 h. This provides proof of principle that transcription factor binding sites can be predicted from de novo motif analyses of DHS profiles and emphasizes the combination of DHS-seq and bioinformatic analyses as a valuable discovery tool to identify potential novel regulators of biological processes in an unbiased manner.

Intriguingly, we found a high degree of overlap between the binding sites of all transcription factors investigated, with about 1000 transcription factor “hotspots” where all factors, that is, GR, Stat5a, C/EBP β , C/EBP δ , and RXR, bind to the same site. These hotspots are found in remodeled regions, and DNase I accessibility appears to correlate positively with the number of associated transcription factors, with GR being the most deciding factor. Importantly, the hotspots are highly enriched in the vicinity of genes that are induced by the cocktail, whereas other sites with fewer factors associated are less enriched. Again, GR seems to be the most deciding

factor. Thus, these data are consistent with the notion that open chromatin regions are associated with binding of transcription factors that recruit remodeling complexes. Furthermore, the data indicate that many hotspots represent functional enhancers that are already active at the 4 h time point. Most likely several other types of hotspots with different combinations of transcription factors exist (Fig. 3A). These early hotspots would represent key “action points” (i.e., major enhanceosomes) on chromatin where the early adipogenic signals converge through the activation and binding of different subsets of early adipogenic transcription factors to overlapping/adjacent DNA elements.

The Role of C/EBP β in the Establishment of Hotspots

The ChIP-seq profiles of transcription factors at the 4 h time point showed that, in particular, GR and RXR, but also Stat5a, overlap very significantly with C/EBP β (RXR and GR $\sim 93\%$; Stat5a $\sim 65\%$) and to a lesser extent also with δ . This indicates that these two members of the C/EBP family constitute common co-occupying

factors for a number of early adipogenic transcription factors. ChIP-seq profiling showed that C/EBP β binds to a large number of sites already prior to induction of differentiation, whereas binding of C/EBP δ , GR, and Stat5a is considerably more dependent on the adipogenic cocktail. Hotspots could be divided in two different clusters—one where prominent C/EBP β occupancy is already established prior to induction of differentiation, and another where C/EBP β occupancy is significantly increased concomitantly with the binding of the other factors and remodeling of the sites. Interestingly, knockdown of C/EBP β resulted in significantly reduced recruitment of GR, Stat5a, and RXR to most of the hotspots investigated, even if C/EBP β occupancy was increased for many sites. This indicates that C/EBP β may function as a pioneering factor for the establishment of at least a subset of hotspots. It remains to be investigated how C/EBP β is pioneering the subsequent formation of early hotspots. However, recent data have shown that hotspots with C/EBP β binding at time point 0 are highly enriched for active histone marks such as H3K27Ac at this time point (R Siersbæk and S Mandrup, unpubl. data). The central role of C/EBP β in establishment of early hotspots

Figure 3. Adipogenic signals are integrated at transcription factor hotspots. (A) Adipogenic inducers activate multiple early transcription factors, which in a cooperative fashion bind to a subset of accessible genomic regions (i.e., hotspots). Different types of hotspots occupied by distinct subsets of transcription factors seem to exist. (B) The first wave of transcription factors induces late-acting adipogenic factors. Similar to the early factors, these late-acting transcription factors are likely to form different types of transcription factor hotspots integrating late adipogenic signals. GH, growth hormone.

is consistent with its important role in adipocyte differentiation (Tanaka et al. 1997; Tang et al. 2003a).

Cross Talk between PPAR γ and C/EBP α in the Late Wave of Adipogenic Transcription Factors

Based on our data demonstrating the existence of transcription factor hotspots in early stages of adipogenesis, we predict that different classes of hotspots are also present in the late adipogenic phase and in mature adipocytes (Fig. 3B). Similar to the early hotspots, these would integrate a number of adipogenic and metabolic signals on discrete genomic sites. Genome-wide ChIP-chip profiles from the Lazar laboratory demonstrated a high degree of overlap (63%) between PPAR γ and C/EBP α in 3T3-L1 cells (Lefterova et al. 2008). We subsequently documented a similar overlap using ChIP-seq analysis, and we showed that the overlap is conserved in human adipocytes (Schmidt et al. 2011).

Interestingly, comparison of mouse and human binding sites of PPAR γ and C/EBP α at orthologous genomic positions demonstrates that the retention of mouse binding sites in human adipocytes is below 20% for PPAR γ (Mikkelsen et al. 2010; Schmidt et al. 2011; Soccio et al. 2011) as well as C/EBP α (Schmidt et al. 2011). However, there are features that appear to increase the evolutionary pressure to maintain binding sites. These include binding strength (i.e., degree of occupancy) as well as vicinity to genes induced during differentiation. Intriguingly, overlap between C/EBP α and PPAR γ binding also increases the frequency of retention, suggesting an increased functional importance of these sites and/or cooperative mechanisms of binding (Schmidt et al. 2011). Furthermore, overall sequence conservation in the binding region also contributed greatly to retention of binding sites independent of conservation of the C/EBP and PPAR consensus binding sites. This is consistent with cooperative binding of additional transcription factors to these regions.

Cumulatively, these findings indicate a high degree of cooperativity between what has so far been established as the two main adipogenic transcription factors, PPAR γ and C/EBP α . In addition to stimulating the expression of each other (Farmer 2006), these two factors appear to cooperate in the activation of the adipogenic gene program by co-occupying a large fraction of the late adipogenic enhancers, most likely as part of an extensive network that involves multiple other late-acting adipogenic transcription factors. We propose that the activity and mode of action of a particular transcription factor like PPAR γ may differ depending on the composition of the enhanceosome it is part of (Fig. 3B).

Potential Cross Talk between Early and Late Hotspots

As discussed above, the first wave of adipogenic transcription factors activates the next wave of factors including PPAR γ and C/EBP α . In addition, our analyses

revealed that about one-third of all PPAR γ binding sites are already remodeled 4 h following stimulation with the adipogenic cocktail, that is, long before PPAR γ and C/EBP α are expressed. The majority of these early remodeled PPAR γ binding sites are occupied by C/EBP β , suggesting that C/EBP β could function as an early priming factor together with other factors that might occupy these sites. The functional importance of early versus late remodeling is currently unknown; however, it is intriguing that a large fraction of the early putative enhanceosomes appears to be “inherited” by late-acting enhanceosomes. This indicates that early enhanceosomes may prime the formation of late-acting enhanceosomes.

CONCLUDING REMARKS

Our findings presented above suggest that adipogenic signals converge at specific genomic hotspots, many of which are likely to represent important regulatory elements controlling reprogramming of transcription during adipocyte differentiation. Similarly, regions that are co-occupied by two or more transcription factors have been described in several other cell types, where they coordinate cell type-specific transcriptional programs (Chen et al. 2008; Heinz et al. 2010; Lefterova et al. 2010; Biddie et al. 2011; He et al. 2011). The precise molecular mechanisms governing establishment of such hotspots remain elusive, but several models can be envisioned (Aagaard et al. 2011): (1) Pioneering factors remodel the chromatin structure at hotspots to create an open chromatin configuration, which assists subsequent binding of additional transcription factors; (2) transcription factors bind to adjacent motifs and coordinate recruitment of co-factor complexes and the formation of an enhanceosome; (3) cooperating transcription factors alternate in binding to overlapping DNA elements and thus work in a sequential fashion to regulate transcription from hotspots; and (4) one or more of the transcription factors may associate with hotspots via an indirect mechanism involving protein-protein interactions. Such indirect recruitment of transcription factors may involve a classical tethering mechanism or interactions between factors bound at hotspots and factors bound at distal sites through looping of the intervening DNA. In addition to addressing how hotspots are established, it will be of key interest to discern the function of individual factors in the formation of enhanceosomes and in transcriptional regulation from such hotspots. Extensive molecular analyses in combination with gain- and loss-of-function experiments will be required to obtain an in-depth understanding of the cross talk between individual factors at transcription factor hotspots.

ACKNOWLEDGMENTS

The authors thank members of the Mandrup Laboratory for valuable discussions and input to the manuscript. This work was supported by funds from the Danish Natural Science Research Council and the Novo Nordisk Foundation.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2011 76: 247-255 originally published online September 6, 2011

Access the most recent version at doi:[10.1101/sqb.2011.76.010512](https://doi.org/10.1101/sqb.2011.76.010512)

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