Nck2, an unexpected regulator of adipogenesis

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ABSTRACT

The regulation of adipose tissue expansion by adipocyte hypertrophy and/or hyperplasia is the topic of extensive investigations given the potential differential contribution of the 2 processes to the development of numerous chronic diseases associated with obesity. We recently discovered that the loss-of-function of the Src homology domain-containing protein Nck2 in mice promotes adiposity accompanied with adipocyte hypertrophy and impaired function, and enhanced adipocyte differentiation \textit{in vitro}. Moreover, in severely-obese human’s adipose tissue, we found that Nck2 expression is markedly downregulated. In this commentary, our goal is to expand upon additional findings providing further evidence for a unique Nck2-dependent mechanism regulating adipogenesis. We propose that Nck2 should be further investigated as a regulator of the reliance of white adipose tissue on hyperplasia versus hypertrophy during adipose tissue expansion, and hence, as a potential novel molecular target in obesity.

Introduction

Obesity is characterized by an expansion of white adipose tissue that relies on a combination of increases in either adipocyte cell size (hypertrophy) and/or adipocyte number (hyperplasia).\textsuperscript{1} In the latter process, new adipocytes are generated from \textit{in situ} differentiation of existing precursor cells through a process defined as adipogenesis. In obesity, the clinical impact of adipogenesis dysregulation seems complex. While enhanced differentiation of adipocyte precursors could contribute to adipose tissue expansion and thus to the pathogenesis of obesity, hyperplastic adipose tissue expansion appears to be less metabolically detrimental than hypertrophic expansion. Indeed, hypertrophied adipocytes have been associated with metabolic disorders due to their ability to constitutively release higher levels of fatty acids, adipokines and proinflammatory cytokines.\textsuperscript{2,3} These, jointly, contribute to ectopic lipids deposition and to insulin resistance in peripheral tissues, resulting in increased prevalence of obesity-related type 2 diabetes that is observed beyond a certain adipocyte diameter threshold.\textsuperscript{4} Hence, while contributing to adipose tissue mass, overcoming an “adipogenesis blockade” may in fact have protective metabolic outcomes, as therapeutically highlighted by the thiazolidinediones (TZD’s).

Adipogenesis

Adipogenesis is a complex process involving coordinated temporal and spatial control of a precise transcriptional network regulating adipocyte-specific gene expression. Members of the CCAAT/enhancer-binding proteins (C/EBPs) family of transcription factors serve as master regulators in initiating the differentiation transcriptional network. Indeed, C/EBP\textgreek{a} and C/EBP\textgreek{b} are expressed early following the induction of adipogenesis. As a result, the C/EBP\textgreek{a} and peroxisome proliferator-activated receptor \textgreek{y} (PPAR\textgreek{y}) axis is activated and these function together as late-acting master transcriptional activators of most genes defining the terminal adipocyte phenotype.\textsuperscript{5,6} Although C/EBP\textgreek{a} and PPAR\textgreek{y} seem to cooperatively induce transcriptional activation of adipogenic genes by occupying common transcription factor hotspots,\textsuperscript{7} PPAR\textgreek{y} appears to be a dominant actor in promoting adipocyte differentiation. Indeed, PPAR\textgreek{y} ectopic expression was reported to restore adipogenesis in mouse embryonic fibroblasts lacking C/EBP\textgreek{a},\textsuperscript{8} while the latter failed to compensate for the lack of PPAR\textgreek{y}.\textsuperscript{9} Important progress was made during the past decades in identifying the importance and the role of PPAR\textgreek{y} in mediating transcriptional regulation of adipocyte differentiation, including the identification of TZD’s as pharmacological...
PPARγ ligands. Yet, specific temporal dynamics involving various protein-protein and protein-DNA interactions regulating transcripational activity of PPARγ during adipogenesis still remain largely unknown.

**PPARγ regulation**

As an essential factor for adipocyte differentiation, PPARγ has been implicated in multiple steps during the entire differentiation process. Indeed, PPARγ contributes to growth arrest following mitotic clonal expansion in early steps of adipocyte differentiation by inhibiting E2F/DP DNA binding activity, induction of p21, or cyclin D1 expression. In addition, PPARγ is a direct transcriptional activator of most specific genes encoding critical proteins regulating adipogenesis and adipocyte function. Among them, aP2 (FABP4), which is required for transport of free fatty acids, perilipin (PLIN1), which covers the surface of mature lipid droplets in adipocytes and regulates lipolysis, and lipoprotein lipase (LPL), adiponectin (ADIPOQ), CD36 (CD36), glycerol-3-phosphate dehydrogenase (GPD1) and insulin-responsive glucose transporter 4 (SLC2A4), all involved in regulating lipid and glucose metabolism. Playing a “master regulator” role, the transcriptional activity of PPARγ is highly regulated during the adipogenic program by post-translational modifications. Indeed, certain growth factors, cytokines and stress conditions repress PPARγ’s transcriptional activity and impair adipogenesis by inducing the phosphorylation of PPARγ on Ser112 via mitogen-activated protein kinases (MAPK)/extracellular-signal regulated kinases 1 and 2 (ERK1/2), or the stress-activated MAPKs JNK and p38. In contrast, PPARγ phosphorylation on the same site by the cyclin-dependent kinases Cdk7 and Cdk9 promotes PPARγ activity, further illustrating the complexity of the regulation of PPARγ by phosphorylation. Beyond phosphorylation, PPARγ’s transcriptional activity in white adipose tissue may also be repressed by sumoylation, ubiquitination and nitrosylation. Another means of PPARγ regulation involves its association, under either unliganded or liganded states, with proteins that behave as coactivators such as PGC-1α or corepressors such as N-CoR and SMRT. Finally, the subcellular localization of PPARγ clearly determines its transcriptional activity, but surprisingly, little is known about the molecular mechanisms regulating its nuclear-cytoplasmic shuttling. Growth factors or cytokines stimulation exported PPARγ from the nucleus toward the cytosol by direct interaction with the Mitogen-Activated Protein Kinase 1 (MEK1), an upstream activator of ERK1/2 that was already mentioned to negatively regulate PPARγ through Ser112 phosphorylation. In contrast, PPARγ nuclear localization was facilitated by its interaction with nocturnin, a protein encoded by one of the circadian clock genes, further illustrating the importance of protein binding partners in regulating PPARγ nuclear-cytoplasmic transport and biologic function.

**A critical role for Nck2 in regulating adipogenesis**

In a recent study we identified the Src homology (SH) domain-containing protein Nck2 (non-catalytic region of tyrosine kinase 2) as a novel regulator of adipogenesis in vitro, and demonstrated that its absence in mice contributes to the development of hypertrophic adiposity and metabolic syndrome. In addition, we uncovered that among severely-obese humans, Nck2 expression is downregulated in visceral white adipose tissue, while it is upregulated during differentiation of human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes (Fig. 1). These findings jointly suggest that Nck2 may regulate the relative contribution of hypertrophy and hyperplasia to white adipose tissue expansion in mice and humans. Nck2 is a member of a family of adaptor proteins, which also includes Nck1. These 47 kDa cytosolic proteins, although encoded by different genes, share high degree of amino acids identity. Both Nck1 and 2 are devoid of catalytic activity, and are composed of 3 N-terminal SH3 domains and a unique C-terminal SH2 domain. Through their highly conserved SH domains, Nck1 and 2

![Figure 1. Expression of Nck2 in human SGBS (Simpson-Golabi-Behmel syndrome) cells during adipocyte differentiation. Nck2 levels were evaluated in SGBS cells at different time points during differentiation by western blot using a specific antibody that recognized human Nck2 (OriGene TA321585) and total cell lysate normalized for protein content. HSP90 was used as loading control. Upper panel represents western blot from a typical experiment, while the bottom panel represents quantitation of Nck2 expression normalized over HSP90 compared with Day 0 from 3 independent experiments. Data are the mean ± SEM. Statistical significance at least p ≤ 0.05 compared with Day 0 using unpaired Student t-test.](image-url)
function as scaffolds that facilitate assembly of molecular complexes linking signaling from cell surface and endoplasmic reticulum (ER) localized transmembrane receptors to crucial cellular processes, such as proliferation, cytoskeletal reorganization and stress response.\textsuperscript{26-28} Within this context, we identified a major role for Nck in regulating signaling from the ER through direct interaction with IRE1-\(\alpha\) (Inositol-required enzyme 1-\(\alpha\)) and PERK (double-stranded RNA-like ER kinase),\textsuperscript{28-32} 2 fundamental components of the unfolded protein response (UPR), whose dysregulation has been implicated in the development of obesity.\textsuperscript{33} It is noteworthy that obesity creates ER stress inducing sustained activation of the UPR in liver and adipose tissue,\textsuperscript{34,35} potentially through increased production of reactive oxygen species\textsuperscript{35} and impaired \(\text{Ca}^{2+}\) homeostasis.\textsuperscript{36} In obesity, ER stress-induced activation of the UPR is clearly associated with dysregulated function of the adipose tissue by altering insulin sensitivity, cytokines and adipokines secretion,\textsuperscript{37,38} but also downregulation of C/EBP\(\alpha\) and PPAR\(\gamma\).\textsuperscript{39} Paradoxically, transient activation of IRE1-\(\alpha\) and PERK plays an important role in normal adipocyte development: The spliced form of XBP1 (XBP1s), generated by activated IRE1-\(\alpha\) RNAse activity, has been reported to promote C/EBP\(\alpha\) expression through direct binding and activation of the \(\text{Cebpa}\) promoter.\textsuperscript{40} Furthermore, PERK deficiency impaired expression of lipogenic genes and lipid accumulation.\textsuperscript{41} During adipocyte differentiation and in adipose tissue, we found that the deficiency of Nck2 results in increased PERK activity and signaling, correlating with higher expression of lipogenic genes.\textsuperscript{25} PERK activation leads to phosphorylation of the eukaryotic initiation factor 2 \(\alpha\)-subunit (peIF2\(\alpha\)), which reduces overall rate of translation initiation, but simultaneously upregulates expression of transcription factors such as activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP).\textsuperscript{42,43} Through the peIF2\(\alpha\)-ATF4 canonical pathway, PERK critically contributes to fat storage and lipogenesis by inducing lipogenic genes expression during adipocyte differentiation in multiple cell types via ATF4-induced expression of the ER membrane-bound transcription factor sterol element binding 1c (SREBP1c).\textsuperscript{41,44} However, the exact mechanism by which disrupting Nck2-dependent control of PERK in white adipose tissue, thereby promoting adipocyte hypertrophy and white adipose tissue dysfunction and obesity has not been fully elucidated.

By contrast to loss-of-function of Nck2 in mouse 3T3-L1 preadipocyte, which promotes adipogenesis, gain-of-function of Nck2 strongly impairs differentiation (Fig. 2A and B). This is supported by a significant decrease in the induction of the fatty acid

![Figure 2](image_url)

Figure 2. Overexpression of Nck2 in murine 3T3-L1 preadipocytes. A) Equivalent amount of proteins from total cell lysates of 3T3-L1 preadipocytes stably transfected with pcDNA3.1 or pcDNA3.1 encoding Flag-Nck2 were probed with anti-Flag antibody to detect Flag-Nck2 or RasGap antibody as loading control. B) Phase-contrast images (10X) of 3T3-L1 preadipocytes control or overexpressing Nck2 at day 0, 6 and 20 of differentiation. C) \(\text{Cebpa}\) and \(\text{Cebpb}\) mRNA levels determined using qPCR in control and Nck2 overexpressing 3T3-L1 cells at day 15 of differentiation. Shown is the mean \(\pm\) SEM of 2 experiments performed in triplicate. D) Equivalent amount of proteins from total cell lysates of control and Nck2 overexpressing 3T3-L1 cells at day 15 of differentiation were subjected to western blot analyses using indicated antibodies. Shown is a typical experiment performed in duplicate. E) \(\text{Pparg}\) mRNA levels determined using qPCR in indicated 3T3-L1 cells at 15 d of differentiation. Shown is the mean \(\pm\) SEM of 4 independent experiments. **, statistical significant at \(p \leq 0.01\) using unpaired Student \(t\)-test.
binding protein 4 (Fabp4) mRNA and lipid droplet formation.\textsuperscript{25} As described above, induction of PPAR\(\gamma\) is the primary driver of adipocyte differentiation and Fabp4 expression, resulting from the early induction of the transcription factors C/EBP\(\beta\) and C/EBP\(\delta\) during adipogenesis. In Nck2 overexpressing 3T3-L1 preadipocytes subjected to the classical hormonal differentiation protocol, the levels of C/EBP\(\beta\) and C/EBP\(\alpha\) mRNAs are equivalent to the levels found in similarly treated, empty-vector-transfected 3T3-L1 cells (Fig. 2C). In agreement, C/EBP\(\alpha\) protein levels are also unchanged in response to Nck2 overexpression (Fig. 2D), further implying that Nck2 is not involved in regulating early transcriptional events during adipocyte differentiation. However, mRNA and protein levels of PPAR\(\gamma\) are unexpectedly increased in Nck2 overexpressing 3T3-L1 cells (Fig. 2D and E) in spite of their seemingly impaired differentiation (Fig. 2B) and decreased expression of classical PPAR\(\gamma\) gene targets such as perilipin (Fig. 2D). Interestingly, higher expression of PPAR\(\gamma\) may represent an adaptive response to lower PPAR\(\gamma\) activity that may be in fact ascribed to lower level of Fabp4 mRNA,\textsuperscript{25} which could potentially results in low level of Fabp4 (aP2), a condition that has been previously reported to mediate a negative feedback loop on PPAR\(\gamma\) expression.\textsuperscript{45} However, Nck2 overexpression hampered PPAR\(\gamma\) nuclear translocation, as revealed by immunofluorescence (Fig. 3A), strongly suggesting a novel mechanism by which Nck2 may limit adipogenesis. In this perspective, the possibility that the SH domain-containing adaptor protein Nck2 may provide a fine control of adipogenesis via directly or indirectly controlling PPAR\(\gamma\) cytoplasmic retention, is an interesting avenue that remains to be defined (Fig. 3B). Potentially, Nck2 might regulate PPAR\(\gamma\) nuclear-cytoplasmic distribution by directly interacting with, and neutralizing, the adaptor protein downstream of tyrosine kinase 1 (Dok1).\textsuperscript{46} This protein was shown to mediate insulin signaling and

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\caption{PPAR\(\gamma\) cellular distribution in induced control and Nck2 overexpressing 3T3-L1 cells. A) DIC and fluorescent images of control and Nck2 overexpressing 3T3-L1 cells induced for differentiation under the same set up were used to investigate PPAR\(\gamma\) nuclear localization. DIC: light microscopy images at 40X; Blue: nuclear staining with Dapi; Red: PPAR\(\gamma\) immunofluorescence using a specific PPAR\(\gamma\) antibody and a secondary antibody-coupled to Alexa Fluor\textsuperscript{594}; Merge: superposition of Dapi and PPAR\(\gamma\) signals. B) Model of adipogenesis regulation by Nck2. In response to adipogenic cues, loss-of-function of Nck2 promotes adipogenesis by enhancing PPAR\(\gamma\) nuclear translocation, while Nck2 gain-of-function promotes PPAR\(\gamma\) expression, but directly or indirectly, represses PPAR\(\gamma\) nuclear translocation.}
\end{figure}
promote adipogenesis by counteracting the inhibitory effect of ERK1/2 on PPARγ transcriptional activity. Alternatively, given our observation that overexpression of Nck2 limits PERK activation and signaling, impaired adipocyte differentiation in 3T3-L1 overexpressing Nck2 might result from attenuated PERK activation and signaling. A detailed investigation of the role of Nck2 is required to understand its function during adipogenesis and to determine whether like for loss-of-function, Nck2 gain-of-function effects on adipogenesis involve Nck2-dependent regulation of PERK activation and signaling during adipocyte differentiation.

Conclusions and key outstanding questions

Existing pharmacological approaches to promote healthy white adipose tissue and weight loss in obese humans are still limited and accompanied by major secondary effects that dampened their clinical potential. A better understanding of how the SH domain-containing adaptor protein Nck2 regulates white adipose tissue homeostasis may provide a unique opportunity to highlight novel ways to overcome obesity and inspire effective pharmacological strategies to prevent obesity-related chronic disorders. To pursue this objective, additional investigation to further decode the role of Nck2 in adipocyte differentiation and function should address whether a direct protein-protein interaction relationship exists between Nck2 and PPARγ in their endogenous expression levels. Next, it would be important to decipher if manipulating the expression of Nck2 represents a potential avenue to take advantage of the beneficial aspect of adipocyte hyperplasia vs hypertrophy in obesity. Finally, in mature adipocytes, it is worthwhile to elucidate if Nck2 is critical for maintaining the mature adipocyte differentiation state, keeping it fully functional. Providing answers to these questions may be a unique opportunity to elucidate the intricate mechanisms linking WAT biology with obesity and related diseases.

Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

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