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STATE-OF-THE-ART REVIEWS

Regulation of metabolic homeostasis by the TGF-β superfamily receptor ALK7

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ALK7 (Activin receptor-like kinase 7) is a member of the TGF-β receptor superfamily predominantly expressed by cells and tissues involved in endocrine functions, such as neurons of the hypothalamus and pituitary, pancreatic β-cells and adipocytes. Recent studies have begun to delineate the processes regulated by ALK7 in these tissues and how these become integrated with the homeostatic regulation of mammalian metabolism. The picture emerging indicates that ALK7's primary function in metabolic regulation is to limit catabolic activities and preserve energy. Aside of the hypothalamic arcuate nucleus, the function of ALK7 elsewhere in the brain, particularly in the cerebellum, where it is abundantly expressed, remains to be elucidated. Although our understanding of the basic molecular events underlying ALK7 signaling has benefited from the vast knowledge available on TGF-β receptor mechanisms, how these connect to the physiological functions regulated by ALK7 in different cell types is still incompletely understood. Findings of missense and nonsense variants in the *Acvr1c* gene, encoding ALK7, of some mouse strains and human subjects indicate a tolerance to ALK7 loss of function. Recent discoveries suggest that specific inhibitors of ALK7 may have therapeutic applications in obesity and metabolic syndrome without overt adverse effects.

Introduction

The human body is adapted to a Stone-Age lifestyle. Our physiology includes powerful regulatory systems that are optimized to survive periods of food scarcity, limiting catabolic activity to favor the storage of ingested calories. However, in modern sedentary societies, we typically intake more energy than we expend,

Abbreviations
ACTRII, activin receptor type II; AgRP, agouti-related peptide; ALK, activin receptor-like kinase; ALT1, alanine aminotransferase; ASKA, analogue-sensitive kinase allele; ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; BCAT2, branched chain amino acid transaminase 2; BMI, body mass index; BMPRII, BMP receptor type II; BMPs, bone morphogenetic proteins; C/EBPα, CCAAT/enhancer-binding protein alpha; cAMP, cyclic adenosine monophosphate; GDF-3, growth/differentiation factor 3; GnRH, gonadotropin releasing hormone; GSIS, glucose-stimulated insulin secretion; Hadhb, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta; hCG, human chorionic gonadotropin; HFD, high fat diet; IKKε, inhibitor-κB kinase ε; IL-12b, interleukin 12b; Itgax, integrin subunit alpha X; KLF15, Kruppel Like Factor 15; LH, luteinizing hormone; MAF, minor allele frequencies; MPOA, medial preoptic area; MRI, magnetic resonance imaging; NPY, neuropeptide Y; PCOS, polycystic ovary syndrome; PDE3B, phosphodiesterase 3B; PMSG, pregnant mare's serum gonadotropin; POX, proline dehydrogenase; PPARγ, peroxisome proliferator-activated receptor γ; PVN, paraventricular nucleus; SD, standard diet; SVF, stromal vascular fraction; TBK1, TANK Binding Kinase 1; TBRII, TGF-β receptor type II; TGF-β, transforming growth factor-beta; TNFα, tumor necrosis factor alpha; UCP-1, uncoupled protein 1; UCP-3, uncoupling protein 3; WAT, white adipose tissue; WHR, waist-to-hip ratio; β3-AR, beta-adrenergic receptor 3.

leading to abnormal expansion of fat depots and obesity, with its associated comorbidities, including diabetes and cardiovascular disease. While lifestyle modification, such as diet change and exercise, is arguably the preferred strategy to counteract obesity, this is often difficult and, in some cases, not possible. Surgical interventions, such as gastric bypass, can achieve rapid and pronounced results, but these are often not long lasting, and accompanied by side effects. On the other hand, the development of safe and efficacious pharmacological interventions to combat obesity requires a thorough understanding of the molecular mechanisms controlling energy storage and metabolic homeostasis.

Distinct brain centers and a handful of endocrine tissues in the body orchestrate the regulation of energy balance and metabolic functions. In the brain, the arcuate nucleus of the hypothalamus plays a crucial role in the secretion of hormones from the pituitary gland and the regulation of appetite and body weight. In the pituitary, cells expressing follicle-stimulating hormone (FSH) and luteinizing hormone are critical for the control of reproduction. Through their production and secretion of glucagon and insulin, respectively, alpha and beta cells in the endocrine pancreas control the uptake of glucose in different tissues and organs, including muscle, liver, and adipose tissue. In the gut, enteroendocrine cells scattered along the gut epithelium produce a range of hormones that have key roles in the coordination of food digestion and absorption, insulin secretion and appetite. And in fat tissue, adipocytes, long recognized for their role in fat storage, produce and secrete a range of peptides and growth factors, such as adiponectin, leptin, and a variety of cytokines, that have a wide range of metabolic effects in distant organs, including the brain. ALK7 (Activin receptor-Like Kinase 7), a member of the TGF-β receptor superfamily, is specifically expressed by all these tissues with endocrine functions, suggesting functions in systems-level metabolic coordination (Fig. 1).

This paper reviews our current knowledge of the signaling mechanisms and physiological functions of ALK7, mainly as inferred from studies in cell culture systems and genetically modified mice. The recent discovery of human protein-coding variants in ALK7 that correlate with lipid homeostasis and fat distribution suggests important functions for this receptor in human physiology and health.

The TGF-β superfamily: overview of ligands, receptors, and Smad signaling

Arguably one of the largest families of structurally and functionally related growth factors, the TGF-β (Transforming Growth Factor-beta) superfamily comprises over 35 ligands and twelve receptors that combine in different ways to confer a multitude of signals regulating cell behavior, including proliferation, survival, differentiation, and migration. The TGF-β ligands include TGF-βs 1 to 3, activins A, B, and inhibins, over a dozen growth/differentiation factors (GDFs), bone morphogenetic proteins (BMPs), and Nodal proteins [1–3]. Members of this protein family share a stereotypical structural organization forming homo or hetero-dimers of two polypeptide chains of about 120 amino acid residues each, themselves derived by proteolytic cleavage from a larger precursor. In the homodimer, the two chains are arranged around a twofold symmetry axis, forming an elongated, 'cigar-shaped' structure [4,5].

The receptors for TGF-β superfamily ligands are tetrameric complexes comprised of two homodimers of type I and type II subunits, respectively [6,7], although some type I subunits can also form heterodimers. Type I receptors, known as ALK1 to 7, are single-span transmembrane proteins with a Ser-Thr kinase moiety at the C terminus, a flexible juxtamembrane domain, and a relatively short extracellular domain. Most type I receptors have only moderate affinity for ligand and require association with a larger, type II subunit for efficient ligand binding and signaling. Like the type I, type II receptors, for example, TBRII, ActRII, and BMPRII, also contain Ser-Thr kinase activity, but can bind ligand independently. In the tetrameric receptor complex, ligand binding results in phosphorylation by type II receptor kinase of sites in a juxtamembrane region of the type I receptor known as the GS domain, leading to activation of type I kinase activity [8]. As there are many more ligands than type I and type II receptors, TGF-β superfamily receptors typically bind more than one ligand. At present, it is unclear whether different ligands may elicit distinct signaling events from the same receptor complex. A protein called Betaglycan is also referred to as a type III TGF-β receptor [9]. Betaglycan can function as an auxiliary co-receptor moiety, in conjunction with type I and type II receptors, to aid the binding and signaling of TGF-β proteins, particularly TGF-β2. Finally, another set of co-receptors, the Cripto and Cryptic proteins, have also been shown to participate in ligand binding of a distinct subgroup of GDFs as well as Nodal proteins to type I/type II receptor complexes [10–13].

Activated type I receptors phosphorylate a set of intracellular signaling effectors called Smads, either at the plasma membrane, or after internalization in early endosomes [14,15]. Once phosphorylated, receptor-associated Smads (i.e., Smad1, 2, 3, and 5) interact with

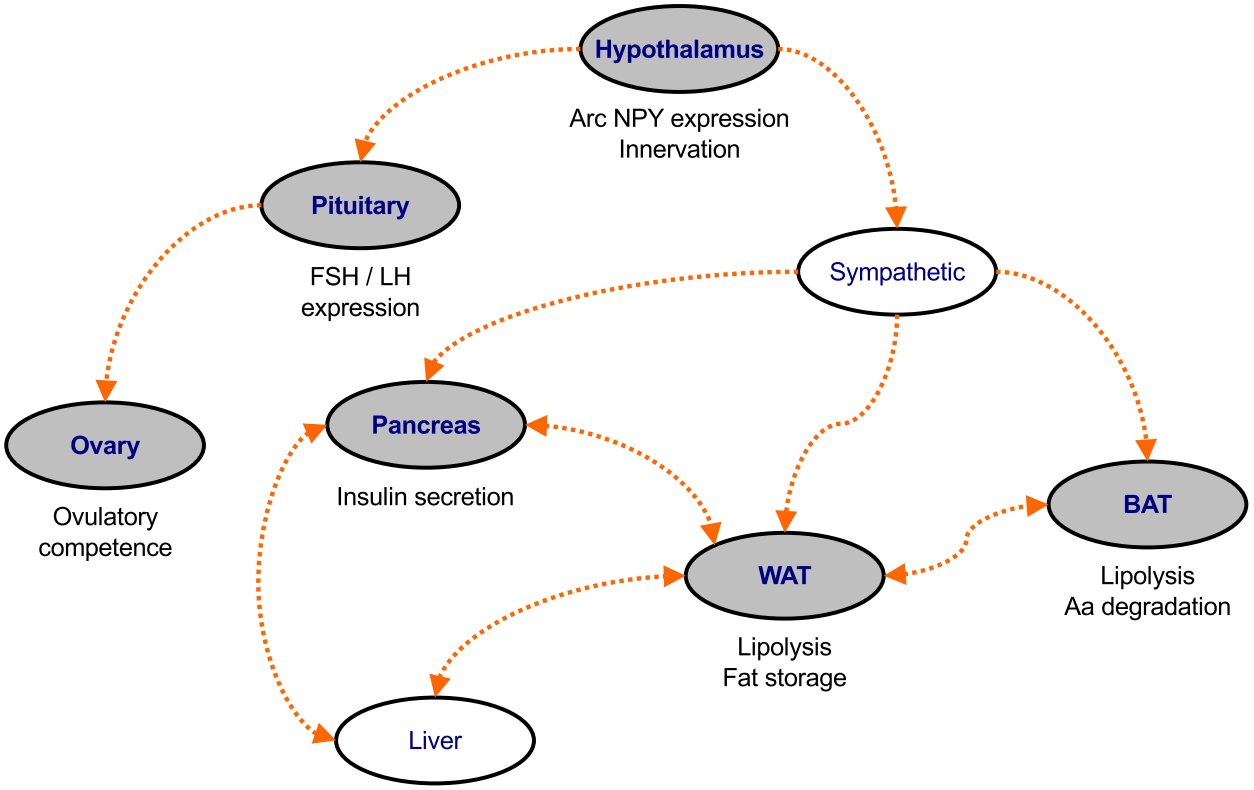

Fig. 1. System-level regulation of metabolic functions by ALK7. Arrows denote direct and indirect routes of tissue/organ influence. Tissues and organs expressing ALK7 are colored in gray, with known ALK7 functions indicated.

a common Smad4 protein forming dimeric or trimeric structures that then translocate to the cell nucleus, where they associate with transcription factors for regulation of target gene expression [7,16,17]. TGF-βs, activins, and a subset of GDFs utilize Smad2 and 3, while BMPs typically signal though Smad1 and 5. Although deceptively simple, this signaling pathway is overlayed by a complex network of regulators, including inhibitory Smads, ubiqitinases, phosphatases, positive and negative transcriptional regulators, chromatin acetylases, and deacetylases, among others [6,7]. In addition to the canonical Smad pathway, Smad-independent signaling mechanisms have also been described, including, for example, members of the MAP kinase, such as TAK1 [18–20]. For a more in-depth review of TGF-β superfamily ligands, receptors, and Smad signaling, the reader is directed to the several review articles cited in this section.

ALK7: an activin receptor in endocrine tissues

ALK7, the seventh and last member to be identified in the type I receptor subfamily, is related to ALK5, the type I receptor for TGF-β1 to 3, and to ALK4, the main type I receptor for activins [21–24]. The gene locus encoding ALK7, termed *Acvr1c,* is located in chromosome 2 of both human and mouse. ALK7 functions in conjunction with the type II receptor ActRIIB and can be activated by a subset of the same ligands that activate ALK4, particularly activin B (but not activin A), GDF1, GDF3, and Nodal [25–28]. Although GDF11 and GDF8 (also known as myostatin) can also interact with ALK7, their preferred receptor has been shown to be ALK5 [29,30]. GDF-1, GDF-3, and Nodal ligands require, in addition to ALK7 and ActRIIB, cooperation from Cripto or Cryptic proteins for efficient signaling [10,27]. ALK7 can signal through Smad proteins 2 and 3 to regulate target gene expression, leading to changes in cell proliferation and differentiation [28,31]. The cytoplasmic domain of ALK7, particularly the GS and kinase domains, shows high sequence identity (over 80%) to those of ALK5 and ALK4 receptors, indicating that these receptors are likely to share very similar, if not identical, signaling mechanisms. In line with this, the kinase activities of ALK4, 5, and 7 can all be inhibited by the same set of small molecules, of which SB431542

is one of the most commonly used in research studies [32]. In the extracellular domains, however, these receptors are very different (about 30% identity), in agreement with their distinct ligand binding specificities. ALK7 is redundant with ALK4 in meditating the functions of Nodal, a protein that is critical for gastrulation and mesoderm formation [33–35]. However, unlike ALK4, ALK7 is dispensable for embryogenesis and left-right patterning in mice. Mice with a constitutive, global knock-out of ALK7 are viable, fertile, and live a normal life under laboratory conditions [36]. In fact, it was recently found that a variety of the common laboratory mouse strain BALB/c carries a missense mutation in the ALK7 kinase domain that renders the receptor inactive [37].

Compared to other TGF-β superfamily receptors, ALK7 expression is much more restricted to specific tissues and cell types with a predominantly endocrine or neuroendocrine function (Fig. 1). Early studies found abundant expression of mRNA encoding ALK7 in adipose tissue, gastrointestinal tract, ovary, prostate, pancreas, and few brain nuclei, notably cerebellum [21–24]. With exception of the arcuate nucleus of the hypothalamus (see section below on reproduction regulation), the function of ALK7 in the brain has not yet been explored. Later studies delineated the expression of ALK7 within these tissues in greater detail. In the pancreas, ALK7 is expressed by all major islet cell types, including glucagon- and insulin-expressing alpha and beta cells, respectively, but not in the exocrine pancreas [38]. In the gut, cells at the bottom of small intestine crypts, presumably Paneth cells, were found to express ALK7 [36]. In the reproductive axis, ALK7 was found in subsets of steroidogenic factor-1 (SF1)-expressing granulosa cells in the ovary, FSH gonadotrophs in the pituitary, and neuropeptide Y (NPY)-expressing neurons in the arcuate nucleus of the hypothalamus [39]. In adipose tissue, ALK7 is expressed by fully differentiated adipocytes in all fat depots so far investigated, including subcutaneous and visceral white adipose tissue (WAT) [23,25,40], as well as brown adipose tissue (BAT) [21,41].

At the time of this writing, there are approximately 120 papers in PubMed featuring ALK7 in their title or abstract. A sizeable fraction of these are based on *in vitro* studies, often using cell lines, in which ALK7 was either over-expressed or pharmacologically inhibited, mostly reporting effects on apoptosis or cell proliferation of alleged relevance to cancer. As the primary focus of this review is on physiological functions of ALK7 in metabolic regulation, as inferred from *in vivo* studies in gene-targeted mice, that literature will not be reviewed in any detail here. Although a majority of those studies report pro-apoptotic and/or anti-proliferative effects of ALK7, or its ligand Nodal, in a variety of cancer cells (e.g., [28,42–46]), others report opposite effects (e.g., [47–50]). Anti-metastatic and pro-metastatic effects have also been reported [49,51,52]. A common limitation of many of these studies is that biological effects were being attributed to ALK7 based on over-expression or pharmacological manipulations based on ligands and compounds that target multiple TGF-β superfamily receptors. Due to the similarities between ALK7, ALK4, and ALK5 discussed above, over-expression of ALK7 will result in activation of pathways that are common to all these receptors, hence not possible to exclusively ascribe to endogenously expressed ALK7. Several microRNAs with effects on proliferation, apoptosis, or invasion of cancer cells have also been reported to target the mRNA encoding ALK7, including miR-382 and miR-548c-3p [53], miR-23b [43], miR-454 [54], and miR-376c [55]. However, these microRNAs have multiple target genes, including other TGF-β superfamily receptors [56], and hence, the specific contribution of ALK7 to the effects reported following microRNA overexpression or knock-down remains unclear. In closing, while pro- and anti-tumorigenic roles of ALK7 in several cancers warrant further investigation, it should be noted that studies of normal mammalian physiology based on gene-targeted mice lacking ALK7 expression, either globally or in specific tissues, have so far not reported abnormal cell growth or apoptosis during development and organogenesis.

ALK7 function in pancreatic islets and insulin secretion

Although pancreas had been recognized as a prominent site of ALK7 expression since the discovery of the *Acvr1c* gene [21,26,31,57], it was not until knock-out mice lacking functional ALK7 were examined that the role of this receptor in β-cell function and insulin secretion begun to be appreciated. Our laboratory found that mice with a constitutive, global knock-out mutation in the *Acvr1c* locus developed hyperinsulinemia (2-fold increase in plasma insulin levels) already at 2 weeks of age; and by 12 months, plasma insulin levels were threefold higher than wild-type littermate controls [38]. Signs of insulin insensitivity (in the C57/BL6 background) were found at 2 and 5 months of age; glucose intolerance appeared at 5 months of age. Liver steatosis started at 6 months of age in these mice and was very pronounced by 12 months. At this age, pancreas islets were significantly enlarged, with a few

islet cells displaying Ki67 immunoreactivity, indicating cell proliferation. Mutant mice lacking ALK7 showed normal pancreas organogenesis, and since hyperinsulinemia preceded all other abnormalities by several months, this was considered the most likely primary consequence of the lack of ALK7. Although pro-apoptotic effects of ALK7 cannot be ruled out, the enlarged islet phenotype observed at older ages is more likely due to ensuing insulin resistance caused by the chronic hyperinsulinemic state of the mutant mice. In agreement with a defect in β-cell function and insulin release, islets from mice lacking ALK7 showed enhanced release of insulin under sustained glucose stimulation, indicating that ALK7 negatively regulates glucose-stimulated insulin secretion (GSIS) in β-cells [38]. Intriguingly, activins showed opposite effects on Ca^{2+} signaling in islet cells, with activin A increasing, but activin B decreasing, glucose-stimulated Ca^{2+} influx. In line with this, mutant mice lacking activin B (*Inhbb*$^{-/-}$) showed hyperinsulinemia comparable with that of *Alk7* knock-out mice, but double mutants showed no additive effects, suggesting that ALK7 and activin B function in a common pathway to regulate insulin secretion. Activin B is endogenously produced by islet cells, and its expression was positively regulated by glucose, suggesting a negative feedback loop controlling insulin secretion from β-cells. These studies indicated that ALK7 regulates the functional plasticity of pancreatic islets, negatively affecting β-cell function by mediating the effects of activin B on Ca^{2+} signaling.

Subsequent studies looked more directly at the role of activin B in the regulation of glucose-stimulated insulin secretion *in vivo* using mice with a null mutation in the *Inhbb* gene, encoding activin B. A study from 2012 by Bonomi *et al.* [58] found no difference between mutant and wild-type mice in either fasting glucose or insulin levels, glucose tolerance, insulin sensitivity, or insulin secretion. This study was conducted in outbred mice and treated islets with activin B several hours prior to glucose stimulation, a major difference with other studies. A subsequent study from our laboratory, performed in *Inhbb*$^{-/-}$ mice in an inbred C57/Bl6 background, found that mice lacking activin B displayed elevated serum insulin levels and increased GSIS, both *in vivo* and in perfusion experiments performed on isolated islets [59], in agreement with the phenotype of mice lacking the ALK7 receptor. Interestingly, *Inhbb*$^{-/-}$ mice have been shown to display some defects (e.g., failure of eyelid fusion) in the C57/Bl6 background but not in Sv129 or hybrid backgrounds [60], suggesting a strong influence of genetic background on activin B functions. Elevated insulin and reduced glucose in plasma were observed already 2 days after birth, suggesting a primary defect in *Inhbb*$^{-/-}$ mice. Importantly, injection of a soluble activin B antagonist (i.e., RAP-435, a recombinant fusion protein derived from an optimized human ActRIIB extracellular domain [61]) phenocopied these changes in wild-type mice. Moreover, exogenous activin B rescued the effects of the *Inhbb*$^{-/-}$ mutation on glucose-stimulated insulin secretion, and negatively regulated glucose-stimulated ATP production in wild-type islets. Electron microscopy studies revealed increased mitochondrial number in β-cells from *Inhbb*$^{-/-}$ mice. Intriguingly, activin A displayed opposite effects to activin B on ATP synthesis, highlighting the antagonistic actions of the two activin ligands [59]. Mechanistically, it was found that the downstream mediator Smad3 responded preferentially to activin B in wild-type pancreatic islets and in MEPI and INS-1 β-cell lines, while Smad2 showed a preference for activin A, indicating distinct signaling effects of the two activins [59]. In line with this, adenovirus-mediated overexpression of Smad3, but not Smad2, decreased GSIS in pancreatic islets of wild-type mice. These results suggest a tug-of-war between activin ligands in the regulation of insulin secretion by β-cells. The mechanisms by which Smad2 and Smad3 are differentially activated by activins A and B, as well as those underlying the differential effects of the two Smad proteins on glucose-stimulated insulin secretion remain to be elucidated.

ALK7 function in reproduction regulation

Activins and inhibins were first functionally characterized for their ability to respectively stimulate or inhibit secretion of FSH from the anterior pituitary [62,63], and are among the most important signals regulating reproduction. Activin signaling affects the reproductive axis at multiple levels, including hypothalamus, pituitary, gonads, and placenta [64]. Female mice lacking activin B have a marked impairment in reproductive function [60]. The role of ALK7 in female reproductive function was investigated in global *Acvr1c*$^{-/-}$ knock-out mice [39]. As indicated earlier, ALK7 is expressed in different tissues along the reproductive axis, including hypothalamus, pituitary gland, and ovary. Mouse females lacking ALK7 showed delayed onset of puberty, abnormal estrous cyclicity, and abnormal diestrous levels of FSH (50% of wild-type) and LH (30% higher than wild-type) in serum at adulthood. In agreement with this, basal LH secretion was higher in pituitary cultures derived from *Acvr1c*$^{-/-}$ knock-out mice, indicating a cell-autonomous

abnormality in pituitary cell function. Mutant ovaries showed premature depletion of follicles, oocyte degeneration, and impaired responses to super ovulation induced by exogenous gonadotropins PMSG and hCG, indicating impaired ovulatory competence in the mutants. These results indicate that ALK7 may mediate some of the effects of activin B, the most abundant activin in developing ovary, on follicular pool maintenance at puberty, the time when massive degradation occurs and developmental competence is acquired. Interestingly, the ovaries of adult mice lacking ALK7 did not show any signs of hypertrophy, cyst incidence, or tumor formation, suggesting that ALK7 does not have a pro-apoptotic function *in vivo*, as it had been indicated by previous *in vitro* studies using overexpression of a constitutively active ALK7 mutant in epithelial ovarian cancer cell lines or in cultures of dissociated granulosa cells [65,66]. On the other hand, the paucity of growing follicles and corpora lutea in ovaries of *Acvr1c* mutant females resembled the phenotype of transgenic mice over-expressing the inhibin α subunit, which is known to oppose several of the functions of activins [67]. ALK7 may thus contribute to establish the physiological balance between activins and inhibins in the ovary [39].

Analysis of the hypothalamus of *Acvr1c*$^{-/-}$ knock-out female mice showed reduced expression of *Npy* mRNA and lower numbers of *Npy*-expressing neurons than wild-type controls in the arcuate nucleus, with the anterior and mid regions mostly affected. A similar reduction in *Npy* mRNA levels was also reported in the arcuate nucleus of female mice lacking activin B [39]. Interestingly, it has been shown that intra-cerebro ventricular (icv) administration of insulin can reduce arcuate *Npy* transcription [68]. As both *Acvr1c* and *Inhbb* mutant mice have increased serum insulin levels (see previous section), it is also possible that their hyperinsulinemia contributes to the reduced levels of *Npy* mRNA expression in the arcuate nucleus of these mutants. The deficits in arcuate NPY expression translated into a marked reduced innervation by NPY (75% reduction) and AgRP (40% reduction) afferents to the hypothalamic medial preoptic area (MPOA), a pathway that plays an important role in regulating the production and release of gonadotropin releasing hormone (GnRH). Although the MPOA is thought to receive NPY innervation from several sources, the parallel reduction in AgRP fibers, all of which originate from NPY arcuate neurons, indicated impaired arcuate NPY/AgRP innervation of GnRH neurons in *Acvr1c* mutant mice. Interestingly, no reduction in NPY fiber density was observed in the mutant paraventricular nucleus (PVN) [39], a second major rostral target of arcuate NPY fibers, suggesting that ALK7 plays a distinct role in hypothalamic wiring, in addition to regulating arcuate *Npy* mRNA levels and neuron number. It is currently unclear whether different subsets of arcuate NPY neurons project to MPOA and PVN or whether the same neurons send collaterals that terminate in both targets. It is therefore possible that ALK7 regulates the differential innervation of these nuclei by mediating neurotrophic functions of MPOA-derived activins.

ALK7 function in white adipose tissue: a rheostat for energy storage

High expression of mRNA encoding ALK7 in adipose tissue was already reported by several of the early studies that identified this receptor [21,23]. A semi-quantitative RT-PCR study in the pre-adipose cell line 3T3-L1 and in primary stromal vascular fraction (SVF) of inguinal and visceral fat pads found that levels of mRNA encoding ALK7 are undetectable or very low in SVF and pre-adipocytes, but raise during later stages of adipogenesis, suggesting that ALK7 is predominantly expressed in mature adipocytes [69]. In our own studies, using quantitative real-time PCR during adipogenic differentiation of SVF from inguinal WAT [Lee and Ibáñez, in preparation], we found that expression of mRNA encoding ALK7 is negligible in SVF (which contains mesenchymal progenitors, pre-adipocytes, endothelial cells, pericytes, T cells, and macrophages), and only begins to rise after 2 days of differentiation *in vitro*, reaching maximal levels by 6 days (Fig. 2). Interestingly, this time-course overlaps with that of *AdipoQ* mRNA (Fig. 2), encoding adiponectin, one of the principal adipokines produced by fully differentiated adipocytes, with important functions in the regulation of glucose and fatty acid metabolism [70]. Levels of the mRNA encoding Leptin, a major peptide hormone released by highly mature adipocytes, only rise at later time points of adipocyte differentiation, concomitant with lipid accumulation (Fig. 2), These results indicate that ALK7 expression parallels adipocyte differentiation and becomes maximal at the onset of lipid accumulation by mature adipocytes. Studies using SVF derived from interscapular BAT have yielded similar results [41], confirming that mature adipocytes are the main, if not the only source of ALK7 expression in both WAT and BAT fat depots.

A role for ALK7 in fat accumulation

The first evidence of the functional involvement of ALK7 in adipocyte physiology and fat accumulation

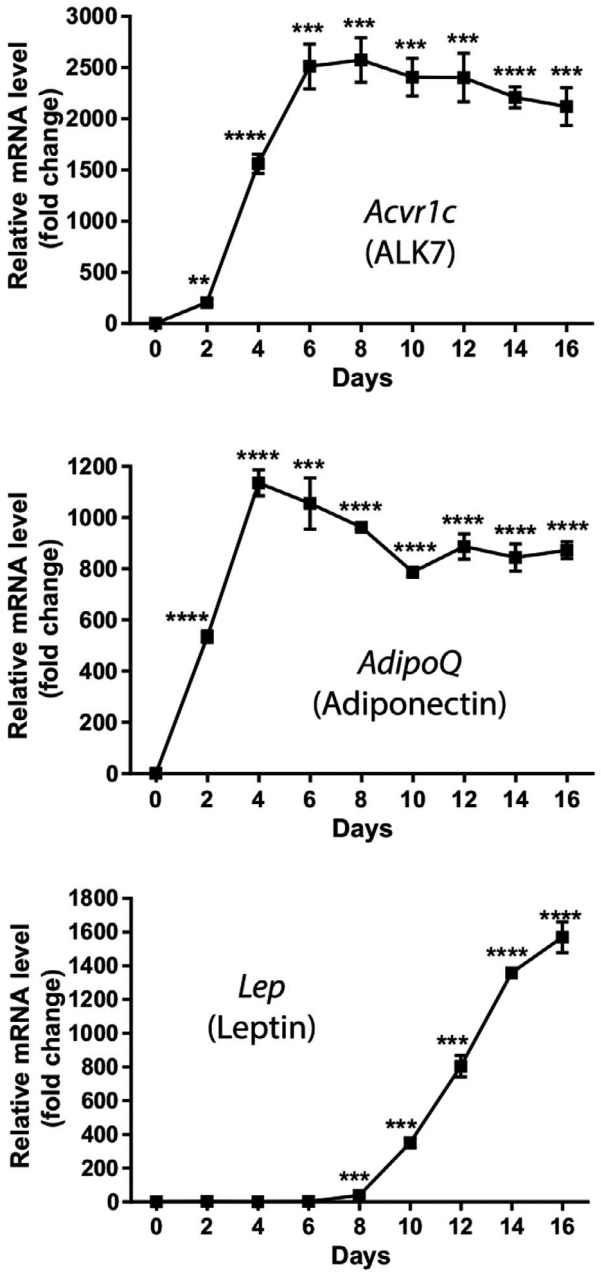

Fig. 2. Expression of *Acvr1c* mRNA, encoding ALK7 (top), *AdipoQ* mRNA, encoding adiponectin (middle), and *Lep* mRNA, encoding leptin (bottom) during *in vitro* differentiation of SVF cells isolated from inguinal WAT. Values are expressed as mean ± SEM. $N = 6$. $^{**}P < 0.01$; $^{***}P < 0.001$; 2-way ANOVA. Adapted from Lee and Ibáñez [in preparation].

was obtained from studies in global knock-out mice initiated at our laboratory. Mutant mice lacking ALK7 showed reduced diet-induced weight gain and fat accumulation when subjected to a high fat diet during 14 weeks [25]. Food consumption was not different from wild-type controls, and no difference in body weight was observed when the mice were fed normal chow diet, indicating a specific defect in fat accumulation during a situation of nutrient overload. Quantification of total body fat by MRI revealed a reduction of 25% in the mutants on high fat diet. Moreover, adipocytes in epididymal fat pads of mice lacking ALK7 were also smaller in size, indicating reduced lipid accumulation in adipose tissue. This study left unclear whether ALK7 affected fat accumulation cell-autonomously in adipose tissue or through other sites, and whether its effects on adipose tissue physiology were developmental or homeostatic, via acute regulation of adult cell function. In a subsequent study, our laboratory generated and studied conditional mutant mice lacking ALK7 specifically in adipose tissue [71]. This paper showed that fat-specific disruption of ALK7 signaling attenuated weight gain and fat accumulation under a high fat diet. Adipocyte cell size was also significantly reduced in these mutants without any changes observed in lean mass. As this study used, the $Ap2^{CRE}$ line of transgenic mice to drive deletion of kinase-encoding exons in the *Acvr1c* gene, a concern arose at the time due to reports indicating ectopic recombination driven by this transgene in cells other than adipocytes, particularly adipose tissue macrophages and selected neuronal subpopulations in the brain [72,73]. However, no *Acvr1c* mRNA expression could be detected in macrophages, and mice lacking ALK7 in the nervous system, generating using the $Nestin^{CRE}$ driver, showed no differences in weight gain or fat accumulation under either normal or high-fat diets [71]. In agreement with reduced fat mass, mice lacking ALK7 in adipocytes had markedly reduced plasma levels of leptin, but, unlike global knock-outs, displayed normal insulinemia, in line with the idea that ALK7 regulates insulin secretion cell autonomously in pancreatic islets. Also different from global ALK7 knock-out mice, the conditional mutants showed glucose and insulin tolerance similar to control mice under high fat diet. Metabolic profiling of these mutants indicated increased energy expenditure and oxygen consumption but normal food intake. Interestingly, mitochondria biogenesis, as measured by mitochondrial DNA content, citrate synthase activity and ATP production, was significantly increased in WAT of the mutants under high fat diet, indicating elevated basal metabolic rate. This was paralleled by increased levels of mitochondrial markers, including PGC-1, a master regulator of mitochondrial biogenesis [74], mitochondrial uncoupling protein 3 (UCP-3), cytochrome C, and Hadhb, a key mitochondrial enzyme for oxidation of long chain fatty acids [75]. In line

with these findings, fatty acid oxidation activity was enhanced in the adipose tissue of fat-specific ALK7 mutant mice.

ALK7 regulation of lipolysis and catecholamine sensitivity in mouse and human adipocytes

Activation of β-adrenergic receptors by catecholamines is the major regulatory pathway of fat mobilization during starvation and exercise. Mice lacking all three types of β-adrenergic receptors become massively obese on high fat diet without showing increased in food intake [76]. Conversely, treatment with β-adrenergic agonists induces mitochondria biogenesis in adipose tissue and reduces fat mass [77], resembling some of the phenotypes observed in ALK7 mutant mice. In rodents, as well as humans, obesity is associated with blunted responses to adrenaline in adipose tissue, including decreased lipolysis and lipid oxidation [78–80], but the mechanisms linking nutrient overload to catecholamine resistance had been unclear. Mutant mice lacking ALK7 in adipocytes showed enhanced sensitivity to the β-adrenergic agonist CL316243, a specific agonist of β3-adrenergic receptor (β3-AR)—the most abundant β-adrenergic receptor in mouse adipose tissue [71]. Following a high fat diet, both basal and CL316243-stimulated levels of plasma free fatty acids, a measure of adipose tissue lipolysis, were elevated in the mutant mice compared to controls, indicating increased catecholamine sensitivity in the mutants. Was this due to the reduced obesity of these animals, or to a direct effect on catecholamine signaling? Importantly, the conditional mutants also showed enhanced CL316243-stimulated lipolysis when on a regular Chow diet, indicating that their increased sensitivity was a direct effect on the catecholamine signaling pathway. In line with this, levels of mRNA encoding β3-AR were strongly upregulated in epididymal fat tissue of the mutants, both under Chow and high-fat diets. The levels of phosphorylated HSL (hormone sensitive lipase), a key enzyme for triglyceride lipolysis, as well as other phosphorylated substrates of protein kinase A, the key downstream mediator of catecholamine effects, were also elevated in mutant adipose tissue compared to controls. Supporting the cell-autonomy of these effects, stimulation of cultured adipocytes with the ALK7 ligand activin B, but not the ALK4 ligand activin A, strongly repressed mRNA levels of β3-AR in wild-type cells, but not in adipocytes lacking ALK7 [71], indicating the effects were ALK7-specific. In line with these results, activin B also decreased phospho-HSL levels and basal as well as stimulated lipolysis in cultures of both mouse and human adipocytes, suggesting similar functions for human ALK7 as a negative regulator of catecholamine sensitivity and β3-adrenergic signaling.

Serendipitous discovery supports the role of ALK7 as a key regulator of lipolysis and fat accumulation

Independently from the above studies, work from the Izumi laboratory reported similar findings on the role of ALK7 in fat accumulation through a hypothesis-free, if not serendipitous, approach. Back in 2006, these authors had reported on a locus located on mouse chromosome 2 which they thought accounted for the exacerbated adiposity of TSOD mice, a line of mice that spontaneously developed obesity, hyperglycemia, hyperinsulinemia, and diabetes [81]. The locus was found in segregating F2 populations of intercrosses between TSOD mice and what was regarded to be normal BALB/cA mice (a related BALB/c substrain available in Japan), used as control mating strain. From those crosses, the authors characterized a strain of lean mice, termed T.B-*Nidd5/3*, considered to be isogenic with TSOD, except for a ≈10-Mb interval in chromosome 2 derived from BALB/cA mice. As this DNA segment appeared to have rescued the obese phenotype of TSOD mice, it was thus thought to contain gene(s) responsible for the excessive adiposity of TSOD mice. In a follow-up study, T.B-*Nidd5/3* mice were found to be partially resistant to obesity induced by high-fat diet and showed enhanced lipolysis [37], a phenotype that resembled mutant mice lacking ALK7 generated by gene-targeting [25]. Molecular analysis of ≈ 10 Mb region in chromosome 2 of T.B-*Nidd5/3* mice revealed the presence of a nonsense mutation in the *Acvr1c* gene, encoding ALK7, which resulted in a stop codon at position 322 of the protein sequence. Consequently, no mRNA encoding ALK7 could be detected in adipose tissue of T.B-*Nidd5/3* mice, presumably due to nonsense-mediated decay. (Incidentally, although no differences were found in the levels of several other mRNAs encoded in the ≈ 10-Mb DNA interval derived from BALB/cA mice, these genes were not screened for protein coding mutations or polymorphisms.) Thus, the apparently 'normal' BALB/cA strain used as mating control was in fact homozygous for a null mutation in the *Acvr1c* locus, encoding ALK7. Interestingly, TSOD mice carried wild-type alleles of the *Acvr1c* gene and displayed normal levels of mRNA encoding ALK7; thus, the molecular pathogenesis of obesity and diabetes in TSOD mice remains unknown. In a subsequent study, the authors

confirmed increased basal lipolysis in adipocytes derived from ALK7-deficient T.B-*Nidd5/3* mice compared to TSOD mice, but failed to observe enhanced lipolysis after stimulation of β-adrenergic signaling with isoproterenol [82], a discrepancy with previous studies that had shown enhanced catecholamine sensitivity in adipocytes lacking ALK7 [71]. This apparent inconsistency is most likely explained by the use of isoproterenol, a well-known agonist of β1- and β2- (but not β3) adrenergic receptors, commonly used in humans for the treatment of bradycardia. Unlike human adipocytes, the β3-AR is the main driver of catecholamine-stimulated lipolysis in mouse adipocytes, which is why mouse studies use the β3-specific agonist CL316243. Aside of this, the studies by the Izumi laboratory have provided important and independent validation on the role of ALK7 as a critical regulator of fat accumulation and obesity.

Molecular mechanisms of ALK7 regulation of adipose tissue lipolysis

Several studies have begun to shed light on the molecular mechanisms by which ALK7 signaling regulates lipolysis under physiological conditions and in obesity (Fig. 3). Similar to the TGF-β type I receptor ALK5, ALK7 signals by activating Smad proteins 2, 3, and 4 (see section of TGF-β signaling above). Early studies by the Derynck laboratory showed that Smad3 and 4, but not Smad2, proteins can interact directly with members of the CCAAT/enhancer-binding protein (C/EBP) family of transcriptional regulators, thereby repressing their transactivation function [83]. C/EBP proteins are known to be key regulators of adipogenesis and adipocyte differentiation, in part, through regulation of the expression of PPARγ, a master gene for adipogenesis [84,85]. In particular, C/EBPα and PPARγ have been shown to regulate expression of genes encoding key enzymes of lipolysis, including adipocyte triglyceride lipase (ATGL) and HSL [37]. PPARγ is also able to upregulate ALK7 expression, as it would be expected from a marker of differentiated adipocytes. On the other hand, several studies have reported that activation of ALK7 through ligand stimulation or overexpression can suppress PPARγ expression [37,71], a result that might be difficult to reconcile with a role for PPARγ as a positive regulator of lipolysis. As some of these studies were based on overexpression of various constructs in preadipocyte cell lines, such as 3T3-L1, it is unclear whether the effects reported reflect physiological responses of primary adipocytes and, if so, under which conditions. Moreover, it should also be noted that cell populations in adipose tissue (mature adipocytes, preadipocytes, macrophages, etc.) undergo dynamic changes in response to different stimuli and environmental conditions. As the outcomes of many of these manipulations have so far been assessed by analyzing bulk tissue, it is unclear whether they represent real changes in gene expression or else differential expansion or dilution of a particular cell type. On the other hand, this limitation may be resolved by single cell analysis of gene expression in adipose tissue of mutant mice

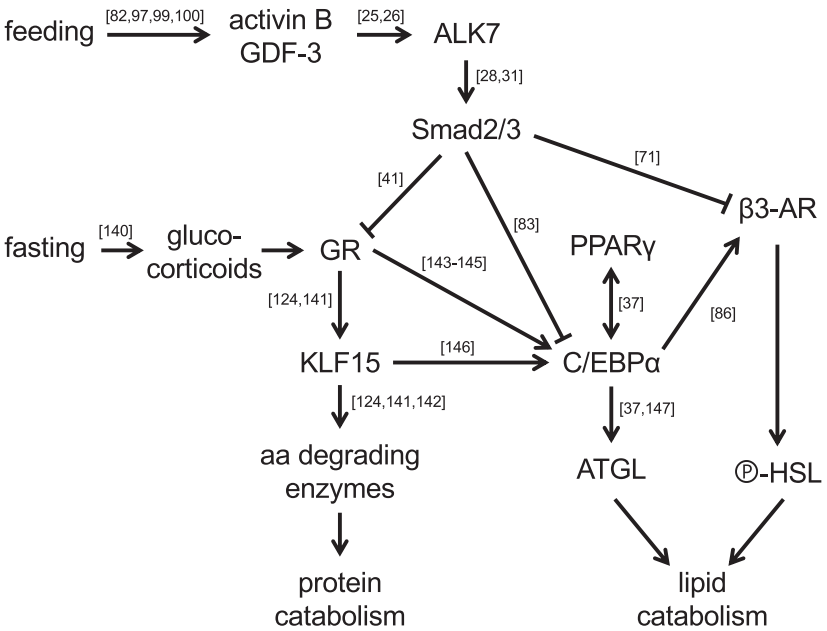

Fig. 3. Signaling pathways implicated in regulation of catabolic activities by ALK7 in BAT. Numbers in brackets indicate references supporting a particular pathway or functional connection: (1) [140]; (2) [124,141]; (3) [124,142]; (4) [143–145]; (5) [146]; (6) [37,147]; (7) [41]; (8) [83]; (9) [71]; (10) [86]; (11) [28,31]; (12) [25,26]; (13) [82,97,99,100]. Adapted from ref. [41].

exposed to different diets and treatments. An additional or alternative mechanism for ALK7 regulation of adipose tissue lipolysis has been proposed based on the ability of ALK7 to induce catecholamine resistance in adipocytes through suppression of β3-AR expression and signaling [71]. In addition to its effects on lipolysis genes, C/EBPα is also required for β3-AR expression during adipogenesis [86], establishing a direct route by which ALK7 signaling may suppress catecholamine sensitivity (Fig. 3). This pathway appears to be independent of PPARγ activity, since neither PPARγ activation with rosiglitazone, nor inhibition with T0070907, had any significant effect on the ability of activin B to suppress β3-AR expression in adipocytes [71].

Effects of acute disruption of ALK7 signaling in adult obesity

Constitutive ablation of ALK7 function, either global or adipose tissue-specific, does not address whether acute disruption of ALK7 activity may affect fat accumulation and energy homeostasis in the adult, and under conditions of pre-existing obesity, similar to the situation normally encountered in the clinic. To begin addressing this question, our laboratory developed a chemical-genetic approach to acutely inhibit the ALK7 kinase in adult mice using synthetic ATP analogues. An analogue-sensitive kinase allele of *Acvr1c* (termed $Alk7^{ASKA}$) was engineered by mutation of two residues in the active site of the ALK7 kinase [71]. Substitution of the 'gatekeeper' residue Ser270 with Gly creates an extra pocket in the ALK7 active site, which can be further expanded by mutating the adjacent residue Leu^{250} to Val. Although the mutations had no effect *per se* on ligand-mediated signaling, they rendered the mutant receptor sensitive to inhibition by ATP competitive inhibitors, such as the 1NaPP1 and 2NaPP1. Analog treatment of adipocytes derived from $Alk7^{ASKA}$ mice showed reduced Smad signaling in response to activin B, while the drug had no effect on wild-type adipocytes. Two-month-old $Alk7^{ASKA}$ mice were subjected to high-fat diet concomitantly with daily injections of 1NaPP1 for 2 weeks. Although $Alk7^{ASKA}$ and wild-type mice gained weight at the same rate on a high fat diet, injection of 1NaPP1 significantly reduced weight gain rate in $Alk7^{ASKA}$ mice but had no effect on wild-type animals [71], suggesting that acute disruption of ALK7 signaling can protect adult mice from diet-induced obesity. In line with this, analog treatment reduced fat accumulation and adipocyte cell size, and increased CL316243-stimulated lipolysis and β3-AR expression and signaling in $Alk7^{ASKA}$ adipocytes. Together, these data indicated that acute disruption of ALK7 signaling in adult mice can uncouple nutrient overload from catecholamine resistance in adipose tissue, resulting in reduced fat accumulation and decreased weight gain on a high-fat diet. However, as these studies inactivated ALK7 prior to diet-induced obesity, they left open the question of whether ablation of ALK7 may have comparable effects in mice that are already obese.

In order to address whether acute disruption of ALK7 signaling can have an effect in adult mice with pre-existing obesity, our laboratory generated a line of mutant mice to conditionally inactivate ALK7 in adipose tissue of adult mice by acute injection of tamoxifen [87]. $Alk7^{flox/flox}$ mice were bred to $AdipoQ^{CreERT2}$ mice, expressing tamoxifen-inducible Cre recombinase under the control of the mouse *Adipoq* locus, driving recombination specifically in fully differentiated adipocytes [88]. In these mice, expression of mRNA encoding ALK7 was reduced by more than 95% two weeks after tamoxifen injection. In these studies, tamoxifen was administered following a 12-week high-fat diet, and animals were initially examined during and at the end of additional 7 weeks on high-fat diet. Disappointingly, disruption of ALK7 had little effect on the weight-gain trajectory of these animals. Loss of ALK7 resulted in a rather small decrease (≈ 2 g) in body weight in mutant mice kept on high fat diet compared to controls. This was accompanied by only modest changes in β3-AR expression, β-adrenergic signaling, and lipolysis in the mutants, indicating that, in addition to ALK7 activity, other factors must contribute to suppress WAT catecholamine signaling in the obese state. It was therefore decided to combine ALK7 ablation with other interventions, including life-style change and anti-inflammatory treatment, that are known to affect different aspects of metabolic function but which, by themselves, have only transient effects in obesity [89–95]. Indeed, ALK7 ablation was much more effective when obese mice were switched to a low-caloric standard diet, paralleled by a robust increase in β3-AR expression, β-adrenergic signaling, lipolysis, and energy expenditure in the mutants [87]. On its own, diet switch had a much more limited and transient effect. Interestingly, however, diet switch led to a significant reduction in the expression of a series of inflammation markers in both control and mutant mice. This suggested that the overt inflammation state that characterizes adipose tissue in obesity may make adipocytes refractory to the effects of ALK7 ablation on adrenergic signaling and energy expenditure. Direct support for this notion was obtained from studies using Na-salicylate. As expected, salicylate treatment reduced the expression of inflammation markers in

adipose tissue of obese mice, although it did not have any effect on adrenergic signaling or lipolysis on its own [87]. On the other hand, it synergized very strongly with ALK7 deletion, resulting in enhanced β3-AR expression, increased lipolysis and reduced adipose tissue mass and body weight gain, even under sustained high caloric intake. Previous studies had shown that ALK7 ablation in adipose tissue of lean mice prevents diet-induced upregulation of several molecules associated with inflammation, including TNFα (tumor necrosis factor alpha), Itgax (Integrin Subunit Alpha X), and IL-12b (interleukin 12b), that are recognized markers of pro-inflammatory M1 macrophages in adipose tissue [71]. In contrast, disruption of ALK7 in mice with pre-existing obesity had by itself very limited effect on the expression of inflammation markers [87]. Inflammatory cytokines, such as TNFα, have been shown to increase the expression of noncanonical members of the IKK kinase family, including IKKε and TBK1, which can in turn phosphorylate and activate the major adipocyte phosphodiesterase PDE3B [96]. This observation provides a possible pathway linking inflammation to catecholamine resistance, as phosphodiesterases antagonize cAMP signaling induced by β-adrenergic receptor activity. In the presence of ALK7, however, anti-inflammatory interventions that reduce phosphodiesterase activity may have limited effects on catecholamine signaling due to low levels of β-adrenergic receptors. Likewise, increased β3-AR expression following ALK7 deletion may not be sufficient to increase lipolytic signaling in the obese state due to high phosphodiesterase activity induced by inflammatory cytokines. These observations suggest that ALK7 signaling and inflammation work together to suppress β-adrenergic signaling in adipose tissue of obese mice, preserving fat mass. Both these pathways need to be inhibited to relieve this suppression and thereby enhance lipolysis and energy expenditure.

Life-style change is arguably the safest and most prevalent intervention used to counteract obesity. However, as difficult as it is to adhere to a severe diet and exercise regime, even more difficult it is to maintain the lost weight over longer periods of time, particularly after the end of a weight-lowering program. A study of participants undergoing an intensive diet and exercise intervention as part of 'The Biggest Loser' televised weight loss competition found that on average 70% of the lost weight was regained 6 years after the competition [90]. Weight loss results in a slowing of resting metabolic rate, a metabolic adaptation that acts to counter weight loss, and a likely contributing factor to weight regain [91]. In agreement with this notion, obese mice that were switched to standard diet and subsequently returned to high-fat diet surpassed the body weight of a parallel cohort that had remained in high-fat diet all the time (Fig. 4). Interestingly, however, mice in which ALK7 had been ablated at the time of the switch to standard diet, were able to maintain a significantly reduced weight upon resuming the high fat diet compared to the control cohort (Fig. 4) [87]. This result suggests that ALK7 ablation helped to sustain the anti-obesity effects of a diet switch, even after return to a high caloric diet regime.

ALK7 ligands in adipose tissue function and homeostasis

Two main ligands have been postulated to induce the activities of ALK7 in adipocytes, namely activin B and GDF-3. Both are able to activate ALK7 signaling in heterologous systems, although GDF-3 requires, in addition to ALK7 and a type II receptor, the GPI-anchored protein Cripto [25,26]. Primary adipocytes respond to activin B in an ALK7-dependent manner [71]. On the other hand, whether primary adipocytes respond directly to GDF-3, and under which conditions, has not yet been clarified. This is in part due to lack of a functionally validated source of purified GDF-3 protein. Both ligands are present in plasma

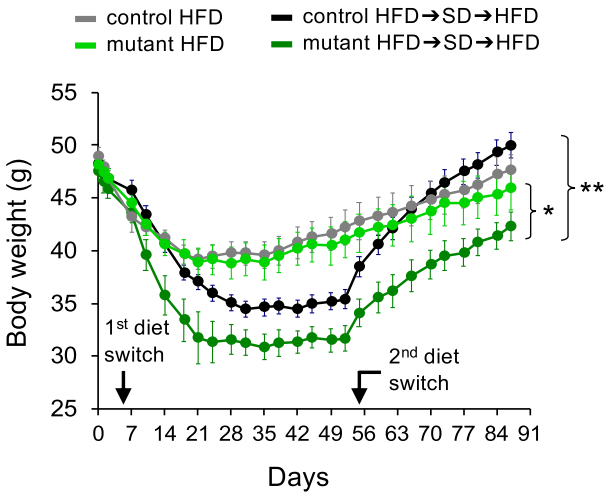

Fig. 4. Acute ablation of ALK7 in adipose tissue of obese mice enhances weight loss after temporary switch to low fat diet. Shown are body weights of obese mice following acute deletion of ALK7 in adipose tissue (day 0, mutant) compared to control mice, either kept in constant high-fat diet (HFD) or after double-switch to standard diet and back to HFD (HFD→SD→HFD) as indicated. Values are expressed as mean ± SEM of weight (grams). $N = 6$ mice per group. $^{*}P < 0.05$: $^{**}P < 0.01$; 2-way ANOVA. Adapted from ref. [87].

[97,98] and may thus affect fat deposition through non-cell-autonomous effects. They are also produced in adipose tissue, where their mRNA expression is prominently increased by high-fat diet and during obesity [82,97,99,100]. SVF cells isolated from lean mice express high levels of *Inhbb* mRNA encoding activin B, which then decline upon adipocyte differentiation, indicating that this ligand is mainly produced by adipocyte precursors, endothelial, or myeloid cells [Lee and Ibáñez, in preparation]. Upon ligand-mediated stimulation of ALK7, mature adipocytes differentiated from SVF isolated from lean mice upregulate *Inhbb* mRNA expression. In agreement with this, SVF isolated from obese mice was shown to express lower levels of *Inhbb* mRNA than mature adipocytes [82]. mRNA encoding GDF-3 has been found in both SVF and differentiated adipocytes, and in both cases, its expression is enhanced during high-fat diet [82,100]. Within the SVF, a series of studies have identified adipose tissue macrophages as the main source of GDF-3 in this fraction [82,100,101].

Due to the marked promiscuities of ligand/receptor relationships in the TGF-β superfamily, it has not been straightforward to determine which, if any, of the activities attributed to activin B and GDF-3 in adipocyte function and adipose tissue physiology are mediated by ALK7, particularly *in vivo*. Activin B can regulate expression of mRNAs encoding β-adrenergic receptors, HSL, and PPARγ in fully differentiated adipocytes derived from wild-type mice, but not from mice lacking ALK7 [71]. It has also been shown to suppress lipolysis in adipocytes derived from the 3T3-L1 cell line [102], although the receptors involved were not characterized in that study. In addition, the second type I receptor for activin B, namely, ALK4, is low expressed in mature adipocytes (unpublished observations). Together, these results support the notion that activin B is one of the endogenous ligands capable of eliciting some of the physiological effects of ALK7 on adipocytes. The case for GDF-3, however, appears to be less clear. The Nodal proteins, including Nodal, mammalian GDF-1, GDF-3, and the GDF1/3 homologue Vg-1 in zebrafish, frog, and chicken, are critical components of a signaling pathway that regulates mesoderm formation in vertebrates [13,103,104]. Nodal utilizes ALK4 and ALK7 to mediate its effects in vertebrate development [27]. Using heterologous systems, we and others have reported GDF-3 signaling mediated by ALK4 and ALK7 receptors in a manner dependent on the co-receptor Cripto [10,25,105], a GPI-anchored protein that is required for the activities of all Nodal proteins [106,107]. Interestingly, GDF-3 overexpression in mice by adenovirus gene transfer has been shown to increase adipose-tissue mass and weight gain under a high-fat diet, but had no effect in a normal chow diet [108], suggesting that GDF-3 may act as an adipogenic factor under a high lipid load. Conversely, global *Gdf3* knock-out mice showed increased lipolysis, reduced adiposity, and displayed resistance to diet-induced obesity [25,101,109], phenocopying the effects of either global or adipose tissue-specific ALK7 ablation [25,71,105]. At face value, and together with the *in vitro* results obtained in cultured cells, these *in vivo* studies, although only correlative, can be taken to imply that GDF-3 elicits anti-lipolytic effects in adipocytes through the ALK7 receptor.

On the other hand, the three studies that identified macrophages as the source of GDF-3 have all proposed different functions and mechanisms for GDF-3 in adipose tissue. Camell *et al.* [101] found GDF-3 expression to be upregulated as part of an inflammatory reaction in adipose tissue macrophages of aged mice. They proposed that macrophage-derived GDF-3 mediates increased expression of monoamine oxidases, driving catecholamine catabolism in macrophages, and thereby limiting catecholamine-mediated lipolysis non-cell-autonomously in adipocytes. However, since ALK7 is not expressed in SVF cells, including adipose tissue macrophages [71], this pathway, centered on a macrophage autocrine loop, would appear to be independent of ALK7. A second study by Bu *et al.* [82] proposed that insulin increases GDF-3 expression in adipose tissue macrophages, which in turn limits lipolysis by acting on ALK7, together with Cripto, in adipocytes. A limitation of this study, however, was the use of the BALB/cA mice as 'ALK7-deficient' mice in comparison with C57/Bl6 animals, regarded as wild-type. As pointed out elsewhere [110], these two lines of mice are likely to differ in a multitude of other loci, in addition to *Acvr1c*. Also, given the hyperinsulinemia of mice with a global inactivation of ALK7 signaling [38], attributing the enhanced lipolysis in ALK7-deficient mice to effects of insulin on GDF-3 expression may be problematic. Finally, a more recent report by Hall *et al.* [100] found GDF-3 expression to be downregulated by 60% in adipose tissue macrophages of knock-in mice lacking phosphorylation at serine 273 of PPARγ. The mutant mice displayed improved insulin sensitivity, mimicking some of the effects of PPARγ agonists, such as thiazolidinedione compounds [111], but showed no differences from wild-type mice in body weight when fed on a Chow diet. This is in agreement with results from our group and others showing effects of GDF-3 ablation on weight gain only under situations of nutrient overload [25,109]. Hall *et al.* [100] also showed that viral mediated overexpression of

GDF-3 in adipose tissue reduced insulin sensitivity, but did not affect body weight in normal Chow diet, which is in line with previous GDF-3 overexpression studies [108]. Looking for a signaling mechanism of GDF-3 action, Hall *et al.* tested GDF-3 activities in HEK293 (human kidney fibroblasts) and C2C12 (mouse myoblast) cell lines, but failed to observe activation of Smad2 and Smad3, effectors of the canonical signaling pathway activated by ALK4 and ALK7 receptors. However, a limitation of such analysis is that ALK7 is not present in these cells and it is also unclear whether they make the essential co-receptor Cripto. As an alternative mechanism, the authors found evidence supporting an idea originally put forward by Brivanlou and colleagues, which proposed that GDF-3 functions as an inhibitor of BMP proteins, possibly, through direct interaction [112]. Intriguingly, BMPs have been shown to positively regulate insulin sensitivity [113]. It should be noted that several studies, including from our own laboratory, have found that GDF-3 is indeed relatively inefficient at activating the canonical Smad2/3 pathway [10,114]. However, in the presence of Nodal, GDF-3 activity is dramatically potentiated in heterologous cells, suggesting a synergistic interaction between these two factors [10]. Direct support for the physiological relevance of this notion was provided in a series of recent papers showing that one polypeptide chain of GDF-3 (or its fish, frog, and chicken homolog Vg-1) can heterodimerize with one of Nodal, forming a heterodimeric molecule that was highly active in mesoderm induction and axis formation in the vertebrate embryo [115–117]. On the other hand, GDF-3 homodimers were found to have little, if any, activity. These findings also helped to resolve the long-standing problem of the poor processing of the GDF-3 precursor, which had puzzled the field for a long time [118]. In view of these results, it would be worthwhile to investigate whether Nodal may be collaborating with GDF-3 to activate ALK7 in adipocytes, and mediate the effects attributed to GDF-3 in adipose tissue lipolysis and fat homeostasis.

ALK7 function in brown adipose tissue: preserving energy to resist the cold

Unlike WAT, which functions in the storage of energy, BAT is a thermogenic kind of fat only found in mammals specialized in heat production. The mitochondria of BAT is rich in uncoupled protein 1 (UCP-1), which dissipates chemical energy by short-circuiting the proton gradient across the mitochondrial membrane, a process known as nonshivering thermogenesis [119]. The function of BAT is to defend body temperature against low environmental temperatures. BAT has been shown to originate from a set of dermomyotome-derived precursor cells that also give rise to muscle, but which are distinct from those that generate WAT [120]. Thus, BAT and WAT have not only different functions but also different developmental origins. In spite of this, and similar to white adipocytes, expression of mRNA encoding ALK7 is negligible in BAT SVF and increases during differentiation of brown adipocyte progenitors isolated from the BAT interscapular depot and it is maximal in fully differentiated brown adipocytes [41,121]. Treatments that enhance brown adipocyte differentiation, such as cGMP, also increase expression of mRNA encoding ALK7 [121]. In contrast, ALK7 expression is undetectable in BAT progenitors [41], suggesting that effects attributed to ALK7 in these cells through treatment with activin ligands or ALK7 over-expression [121] most likely reflect the function of endogenous ALK4 receptors, which are abundant in precursor cells of all adipose tissues [Lee and Ibáñez, in preparation].

The physiological role of ALK7 in BAT was addressed in a recent study from our laboratory using mutant mice lacking ALK7 specifically in brown adipocytes [41]. The study used an $Ucp1^{CRE}$ line of transgenic mice to drive deletion of kinase-encoding exons in the *Acvr1c* gene in brown adipocytes. Before summarizing the results of that study, it should be mentioned that a special kind of white adipocyte present in subcutaneous WAT, particularly in the inguinal depot, called 'beige' or 'brite' adipocyte can be induced to express UCP1 upon chronic exposure to cold temperatures, a processes sometimes described as 'browning' [122,123]. Thus, upon chronic cold exposure, ALK7 would also have been inactivated in these cells when using the $Ucp1^{CRE}$ line. BAT lacking ALK7 formed and developed normally, and these mice showed normal body weight, fat composition, and energy expenditure on a standard Chow diet and at room temperature [41]. However, a significant reduction in BAT mass was observed after overnight fasting in the mutants, without any effect on WAT, accompanied by a notable decrease in lipid droplet size, indicating exacerbated lipid depletion in BAT lacking ALK7 [41]. In agreement with this, a specific increase in the levels of adipose triglyceride lipase (ATGL), the rate-limiting enzyme of lipolysis, was detected in BAT total lysates and lipid droplets upon fasting, indicating increased fat catabolism in the BAT of fasted mutant mice.

Unexpectedly, BAT lacking ALK7 also showed an abnormal increase in amino acid catabolism upon

nutrient stress [41]. Expression of several enzymes involved in amino acid degradation, including proline dehydrogenase (POX) as well as ALT1 and BCAT2, responsible for degradation of alanine and branched amino acids, was specifically upregulated upon fasting in the mutant BAT. POX, ALT1, and BCAT2 are all targets of KLF15, a key transcriptional mediator of responses to nutritional stress induced by glucocorticoids in liver and skeletal muscle [124,125]. Further analysis of BAT lysates and cultured brown adipocytes showed that ALK7 signaling represses glucocorticoid receptor signaling and KLF15 expression in BAT, a previously unknown effect of ALK7 activity [41] (Fig. 3). This was an important finding, as the mechanisms by which BAT adapts to nutrient stress to maintain body temperature have remained unknown. Although these findings align the fasting response of BAT with those of liver and muscle, KLF15 was found to have a different function in WAT, where it was reported to suppress lipolysis and be itself negatively regulated by fasting [126]. Importantly, mice lacking ALK7 in BAT showed fasting-induced hypothermia upon acute cold exposure. Mutant mice that had been fasted displayed a very rapid drop in body temperature, with over 70% of animals showing temperatures lower than 32 °C after 3 h at 5 °C [41]. In contrast, fed mutant mice showed a normal response to acute cold exposure. It is important to note that acute cold exposure is not long enough to initiate browning in WAT. The results of these studies indicated that ALK7 functions to dampen catabolic activities triggered in BAT upon limited nutrient availability. Under nutrient stress, catabolic functions such as lipolysis and amino acid degradation become abnormally amplified in brown adipocytes lacking ALK7, leaving the tissue energetically unable to cope with the demands imposed by low ambient temperatures.

ALK7 function in human physiology

Unlike most other receptors in the TGF-β superfamily, loss of ALK7 does not compromise development or viability in mice (although female mice lacking ALK7 are subfertile, as discussed above), suggesting that mutations affecting ALK7 function may be tolerated in the human population and perhaps contribute to neuroendocrine and metabolic phenotypes. Indeed, several missense and nonsense variants have been found in the human *ACVR1C* gene using independent sets of exome sequence data from the MIGen, ARIC, and T2D-GENES Consortium studies [127]. Moreover, 18 of 66 720 individuals (0.027%) of European ancestry analyzed carried an early stop codon (Leu32Ter) in *ACVR1C* [128], further indicating a tolerance to ALK7 loss of function in humans. Together, these results suggest that therapeutic inhibition of ALK7 may not have adverse on-target effects.

Several of the reproduction and metabolic defects found in female mice lacking ALK7 show similarities with abnormalities observed in patients suffering from polycystic ovary syndrome (PCOS), including abnormal estrous cyclicity, ovulatory dysfunction, LH hyper-secretion, increased LH/FSH ratio, hyperinsulinemia, and insulin resistance [129]. A genetic variant that has been consistently associated with PCOS in different studies is present in *ACVR2A*, the gene encoding the human activin type IIA receptor [129], indicating that abnormal activin signaling may contribute to PCOS. Thus, gene variants in *ACVR1C* may represent risk factors for human PCOS.

As in mice, expression of activin B is upregulated in adipose tissue in human obesity [130]. The modest reduction in total *ACVR1C* mRNA levels observed in human adipose tissue of obese individuals compared to lean subjects [130] may be due to enhanced proliferation of adipocyte precursors (which lack ALK7) in adipose tissue. On the other hand, it would be largely compensated by the many fold induction of its ligands, indicating that, overall, ALK7 signaling is expected to be enhanced during obesity in humans, as it is in mice.

Two studies published in 2019 reported DNA variants in the human *ACVR1C* gene, which correlated with altered body fat distribution. Emdin *et al.* [127] conducted an exome-wide association study of coding variants in the UK Biobank (encompassing data from 405,569 individuals) with the goal to identify variants that correlated with changes in waist-to-hip ratio (WHR) adjusted for body mass index (BMI) and that protected against type 2 diabetes. They identified 4 variants in the *ACVR1C* gene, 3 of which changed the amino acid residue in the ALK7 protein, and one in a noncoding region. The variants were relatively rare, with minor allele frequencies (MAF) between 0.2% and 7.2%, and they all correlated with reduced WHR/BMI (P values between 10^{-5} and 10^{-17}). Intriguingly, and in agreement with the mouse studies reviewed above, the 4 variants were predicted by algorithms to result in diminished function or expression of ALK7. However, whether they indeed do so, and by which mechanisms, awaits direct experimental demonstration. A second study, analyzed the association of body-fat distribution, assessed as WHR/BMI, with 228 985 predicted coding and splice site variants available on exome arrays in up to 344 369 individuals from five major ancestries [131]. They identified 24 variants, including 15 common and 9 rare (MAF < 5%), some

correlating with higher, some with lower WHR/BMI. Among the latter ones, they found one in *ACVR1C*, namely, Asn150His (MAF 1.1%), which also corresponded to one of the 4 variants identified in the first study. These findings suggest that variants which are predicted to negatively affect ALK7 function or expression lower adiposity and protect against type 2 diabetes, providing human validation for ALK7 was a therapeutic target in obesity and metabolic syndrome.

Unsolved questions and future challenges

ALK7 functions to antagonize catabolic pathways of lipolysis and amino acid degradation to facilitate the preservation and accumulation of energy stores. It represents an evolutionary adaptation that prepares the organism to future periods of food scarcity and starvation, thereby enhancing survival. Food security relaxes the evolutionary pressure to maintain this function, and so ALK7 activity has become less critical, as evidenced by the various deleterious mutations that have accumulated in the *Acvr1c* gene of strains of laboratory mice (e.g., BALB/cA) and modern human populations. These findings suggest that specific inhibitors of ALK7 may have therapeutic applications in obesity and metabolic syndrome without overt adverse effects.

Although the molecular mechanisms by which ALK7 exerts these functions have begun to be delineated (Fig. 3), several questions remain. The mechanism by which ALK7 signaling through Smad3 dampens GSIS in pancreatic islets remains to be resolved. This may be understood in light of ALK7's ability to repress lipolysis, including expression of the key lipolytic enzyme ATGL. It has become clear that lipid signaling contributes to amplify GSIS and that β-cells express ATGL and have an active lipolysis pathway [132–134]. It has also been shown that glucose stimulates lipolysis in β cells [132,134], and β-cell-specific knock out of ATGL disrupts lipolysis and impairs GSIS [135,136]. Although the precise mechanisms by which lipids amplify insulin secretion remain to be elucidated, such a notion would help to align the effects of ALK7 in pancreatic islets and adipose tissue. In the latter, we have a clearer mechanistic picture of the molecular events involved, in which C/EBPα appears to be an essential player. Here, direct binding of Smad3 suppresses the ability of C/EBPα to activate lipolysis genes as well as expression of β3-AR expression during adipogenesis (see section above on ALK7 functions in WAT). It should be noted that some of these connections have only been established in cell lines or *in vitro* systems and so additional evidence supporting their involvement in ALK7 signaling under physiological conditions, including nutrient stress, caloric overload, and cold exposure, still needs to be provided. Also, as mentioned earlier, future studies of the impact of mutations in *Acvr1c* and ALK7 ligands on gene expression in WAT will have to address the dynamic heterogeneity of this tissue by performing single cell analyses under different diet and environmental conditions. As to the endogenous TGF-β superfamily ligands that function through ALK7 to regulate catecholamine sensitivity and lipolysis, the role of activin B appears clear, but there is still work to be done to clarify whether, and if so how, GDF-3 may also utilize ALK7 to mediate its effects on lipolysis in adipocytes. Regarding glucocorticoid signaling, much remains to be known about the mechanisms by which ALK7 affects the expression of glucocorticoid receptor target genes. Smad proteins are known to be avid binders of a large array of transcription factors [6,7], so it is possible that they directly interact with the glucocorticoid receptor to either suppress or otherwise control its function.

As mentioned earlier, although ALK7 is expressed in several brain regions, its function in the nervous system, aside from the arcuate nucleus, remains mysterious. ALK7 is most abundantly expressed in the cerebellum, where it has been detected in both granule and Purkinje cells [21,22]. In addition, several areas in the hippocampus, cerebral cortex and striatum also express significant levels of mRNA encoding ALK7 [21,22]. Although the brain of mice with a global knock-out of ALK7 does not show any gross structural or histological abnormalities [36], detailed studies are warranted to reveal possible defects in specific areas. With the availability of a conditional allele of *Acvr1c*, specific gene targeting in distinct brain regions can now be performed to better address the functions of ALK7 in distinct neuronal subpopulations.

The role of ALK7 in human physiology also needs to come into sharper focus. The discovery of natural variants of the *ACVR1C* gene that correlate with adiposity in human populations opens an avenue for research into the role of ALK7 in fat deposition in humans. Clarifying the mechanisms by which these variants affect ALK7 function will give valuable insights into these questions. Although genetic studies have so far focused on exonic variants, polymorphisms in noncoding regions may affect ALK7 expression and thus warrant investigation of their possible correlation with adiposity and metabolic syndrome. With regard to human reproduction, whether ALK7 plays a role in human PCOS may be clarified through discovery of genetic variants in the *ACVR1C* gene of affected individuals, warranting more effort in this area. Finally,

strategies to inhibit ALK7 safely and specifically without affecting ALK4 or ALK5 need to be developed. The high similarities of the kinase and GS domains of these receptors make it unlikely that conventional kinase inhibitors will be sufficiently specific, leaving the extracellular and transmembrane domains as possible targets, as these are highly divergent from other TGF-β superfamily receptors. Blocking antibodies targeting the ALK7 extracellular domain have been in development but have so far not achieved sufficient potency or specificity for practical use (personal communication). Targeting the transmembrane domain remains a plausible alternative. Recently, different classes of well-known antidepressants have been shown to function, in part, through interaction with the transmembrane domain of the receptor tyrosine kinase TrkB [137], indicating that, contrary to common wisdom, transmembrane domains may indeed represent important targets for drug discovery. Interestingly, it has been proposed that changes in the orientation of receptor subunits mediated by relative rotation of their transmembrane domains contribute to ligand-mediated activation of TGF-β superfamily receptors [138]. Recently, our group identified a small molecule by directly screening for interaction with the transmembrane domain of the TNF receptor superfamily $p75^{NTR}$ [139]. The identified compound displayed receptor-specific activities in cell lines as well as in mice. This pipeline could be adapted to identify compounds acting upon the ALK7 transmembrane domain, which may potentially interfere with dimer or complex formation, activation, or downstream signaling.

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Conflict of interest

The authors declare no conflict of interest.

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