Elucidating the role of plexin D1 in body fat distribution and susceptibility to metabolic disease using a zebrafish model system

Elucidating the role of Plexin D1 in body fat distribution and susceptibility to metabolic disease using a zebrafish model system

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Abstract

Non-communicable diseases (NCDs) such as cardiovascular disease, diabetes and cancer were responsible for 68% of all deaths worldwide in 2012. The regional distribution of lipid deposited within adipose tissue (AT) - so called body fat distribution (BFD) - is a strong risk factor for NCDs. BFD is highly heritable; however, the genetic basis of BFD is almost entirely unknown. Genome-wide association studies have identified several loci associated with BFD, including at Plexin D1 (*PLXND1*) - a gene known to modulate angiogenesis. We recently demonstrated that zebrafish homozygous for a null mutation in *plxnd1* had a reduced capacity to store lipid in visceral AT (VAT) leading to altered BFD. Moreover, we found that type V collagens were upregulated in *plxnd1* mutants, and mediated the inhibitory effect of Plxnd1 on VAT growth. These results strengthen evidence that Plxnd1 influences BFD in human populations, and validate zebrafish as a model to study BFD. However, many pertinent questions remain unanswered. Here we outline potential Plxnd1 mechanisms of action in AT, and describe the genetic architecture at human *PLXND1* that is associated with BFD and NCD susceptibility.

Commentary

Adipose tissues (ATs) regulate energy homeostasis by supplying and sequestering energy-dense lipid in response to fluctuations in energy status. As such, AT provides an organism with energetic stability [1]. Evolutionarily, the energy insurance provided by AT confers tremendous selective advantages for a population when confronted with diverse physiological burdens. However, in modern societies - when energy-dense food is readily available, food consumption is high and physical activity is low - excessive lipid deposition within AT can lead to AT dysfunction and systemic metabolic disturbance increasing risk for non-communicable diseases (NCDs) such as cardiovascular disease, diabetes and cancer. In 2012, NCDs accounted for 68% of all deaths worldwide [2, 3]. Of the 52.8 million deaths globally in 2010, ischemic heart disease and stroke collectively killed 12.9 million (24% of all deaths), 1.3 million deaths were caused by diabetes (2.5%) and 8 million died from cancer (15%). Therefore, understanding factors that influence or predict NCD risk is an important public health challenge.

ATs are highly heterogeneous and deposited in diverse regional locations throughout the body. Regionally distinct ATs have unique molecular and metabolic attributes that influence whole-animal physiology. Accumulation of AT in the upper body (an android BFD) is associated with increased risk for NCDs [4]. Whereas accumulation of AT in the lower body, primarily the legs and thighs (a gynoid BFD) protects from NCD risk [4]. Android BFD is characterized by increases in visceral AT (VAT, AT within the abdominal cavity) and abdominal subcutaneous AT (SAT, AT between skin and muscle), whilst gynoid BFD is characterized by increased gluteal and femoral SAT [4]. Understanding factors that regulate the diverse patterns of BFD within human populations is likely to provide important new therapeutic interventions for NCDs.

Heritability estimates from twin studies suggest that BFD is under extensive genetic control [5, 6]. However, the genetic basis of BFD is essentially unknown. Recently, the Genetic Investigation of ANthropometric Traits (GIANT) consortium performed large-scale meta-analyses of genome-wide association studies (GWAS) to identify loci associated with waist-hip ratio (WHR) – a surrogate measure of android and gynoid patterns of BFD [7, 8]. Intriguingly, GWAS have found WHR-associated loci are independent from more generalized adiposity traits, suggesting that a distinct genetic architecture underlies BFD [9]. GWAS provide an unbiased and comprehensive assessment of genetic loci associated with WHR. However, functional characterization of GWAS loci is essential to identify mechanisms influencing BFD and disease susceptibility.

The rs10804591 single nucleotide polymorphism (SNP) identified in Shungin et al. (2015) encodes a C → A base change, at 3q22.1 ~8kb upstream of the *PLXND1* transcriptional start site (Fig. 1) [7]. The rs10804591 A allele (effect allele, EA) was associated with increased WHRadjBMI (WHR adjusted for BMI) ($P = 2.31 \times 10^{-6}$), increased susceptibility to type 2 diabetes ($P = 1.67 \times 10^{-3}$), increased fasting glucose ($P = 0.048$), increased fasting insulin ($P = 6.08 \times 10^{-3}$), increased blood triglycerides ($P = 9.37 \times 10^{-4}$), decreased Adiponectin ($P = 7.81 \times 10^{-3}$), increased risk for coronary artery disease ($P = 0.018$) and decreased height ($P = 2.53 \times 10^{-5}$). Similar to many of the BFD-associated SNPs, rs10804591 demonstrated a high degree of sexual dimorphism – often exerting a stronger effect in women (Fig. 2) [7]. Further, the effect in males and females appeared different, with males also exhibiting reductions in both waist and hip circumferences (Fig. 2) [7]. Gender differences in adiposity are well known, with females having a higher body fat percentage, and greater gluteal-femoral AT relative to males [10]. BFD is regulated by sex hormones, as evidenced by redistribution of AT towards an android distribution following menopause [11, 12]. rs10804591 EA is common, present at a frequency of 28%, 65%, 28%, 78% within African, American, East Asian, and European populations respectively (1000G Phase 1, a 51% frequency in all individuals). Intriguingly, rs10804591 is located within a predicted promoter flank region 5' of *PLXND1* (asterisk in Fig. 1), suggesting that rs10804591 might regulate *PLXND1* expression. However, the mechanism of rs10804591 action is completely unknown. Importantly, within individuals of European ancestry, rs10804591 is also linked to 41 other common SNPs clustered 5' to *PLXND1* (>0.7 R^2 linkage disequilibrium) (Fig. 1), and many of these linked SNPs also reside in predicted regulatory regions (Fig. 1). Searching the Genotype-Tissue Expression (GTEx) Project revealed that the majority of the 41 SNPs at *PLXND1* were associated with *PLXND1* mRNA changes in whole blood (N = 338). No

associations were found with expression changes in VAT (N = 185); however, this is potentially due to lower sample size. The investigation of functional variants at *PLXND1* is likely to provide exciting new insights into the genetic underpinnings of BFD.

PLXND1 is a multipass transmembrane receptor for a variety of Semaphorin (SEMA) ligands, including SEMA3E [13, 14] and SEMA4A [15]. Binding of SEMA3E/4A to PLXND1 suppresses angiogenesis - the process of new blood vessel formation from existing vessels [13-15], and mutation of Plxnd1 in mouse and zebrafish causes hypervascularization of multiple tissues [16, 17]. Therefore, PLXND1 is a potent anti-angiogenic molecule. The role of angiogenesis is of particular relevance to AT biology as angiogenesis is known to regulate lipid accumulation in AT [18-20], and stimulation of angiogenesis specifically in AT can normalize metabolic disturbances present in obesity [20, 21]. Furthermore, depot-specific angiogenesis has been linked to systemic insulin resistance – a precursor to diabetes [22], suggesting that depot-specific differences in angiogenesis may underlie regional AT expansion and NCD progression. Further, we found that *PLXND1* mRNA was positively associated with hypertrophic morphology in VAT, and was increased in obese type 2 diabetics relative to lean and healthy obese subjects [23].

Prior to analysis of human PLXND1, we turned to zebrafish as a tractable in vivo model system to functionally evaluate the role of PlxnD1 on BFD. Zebrafish possess AT that is morphologically, molecularly, and functionally homologous to mammalian white AT [23-30]. Further the molecular mechanisms governing AT dynamics seems conserved from zebrafish to mammals, as suggested by modulators of nuclear receptors exerting similar effects [31]. Fluorescent lipophilic dyes such as Nile Red and BODIPY can be utilized to visualize and quantify regional AT in live zebrafish (Fig. 3). Analysis of zebrafish homozygous for the functionally null *plxnd1* allele, *fov01b*, revealed an altered BFD, characterized by reduced VAT [23] (both pancreatic and abdominal VAT deposits) [30]. On closer inspection we found *plxnd1* mutant VAT was in a hyperplastic and hyperproliferative state, with an induction of type V collagens in vascular endothelial cells and altered extracellular matrix (ECM) composition [23]. Maintenance of the hyperplastic/hyperproliferative state was dependent on collagen type V alpha 1 (*col5a1*) and conferred resistance to VAT expansion coupled with improved glucose tolerance after exposure to a high-fat diet [23]. These data suggest that the ECM microenvironment can determine the proliferative capacity and growth of VAT, and that vascular endothelial cell-derived Plxnd1 modulates the VAT ECM microenvironment in part through Col5a1 (Fig. 4A). Regarding this mechanism, here we discuss a potential Integrin-mediated pathway by which PlxnD1 may regulate ECM composition.

Integrins are heterodimeric collagen receptors that mediate cross-talk between the cell cytoplasm and extracellular ECM [32]. The Integrin family of genes is comprised of 18 distinct α subunits and 8 β subunits, which can dimerize to produce 24 heterodimeric combinations (the Integrin code). Integrin expression can be regulated transcriptionally [33-37], post-transcriptionally by miRNAs [38], and also at the post-translational level. For example, Integrins can form an inactive ‘closed confirmation’ with low affinity for extracellular ligands, or an active ‘open confirmation’ with high affinity for ligands. Regulation of these states has been well studied,

with multiple regulators identified (e.g., SHARPIN and SHANK) [39, 40]. Although how this form of Integrin regulation impacts adipose tissue is currently unknown. Intriguingly, the metabolic sensor, AMP-activated protein kinase (AMPK), was recently also identified as a regulator of β1 Integrin activity [41]. However, a role for AMPK in regulating adipose ECM and growth is also unknown. Distinct Integrin heterodimers possess different ECM-binding potentials [42], and regulate ECM abundance and composition by modulating collagen synthesis and turnover [43, 44]. Therefore, we hypothesize that PlxnD1 modulates the collagen composition of VAT by regulating the Integrin code displayed on the vascular endothelial cell-surface. It is known that Integrin expression changes during adipocyte differentiation [45], and that overexpression of Integrin α5 in preadipocytes leads to enhanced proliferation and attenuated differentiation [45]. GTPases hydrolyze guanosine triphosphate (GTP), and the GTPase, Rac, is normally downregulated during adipocyte differentiation [45]. Overexpression of Integrin α5 increases Rac activity, suggesting that GTPase levels are critical for preadipocyte proliferation and differentiation [45, 46]. In support, Focal Adhesion Kinase (FAK) plays a central role in Integrin signaling and is essential for adipose expansion [47, 48]. GTPases control many cell functions, including deposition and maintenance of Integrins on the vascular endothelial cell surface [49-53]. Plexin receptors are well known to regulate GTPase activity via their intracellular GTPase activating-protein (GAP) domain [54], and recent studies demonstrated that binding of SEMA3E to PLXND1 in Human Umbilical Vein Endothelial Cells (HUVECs), inactivated the GTPase activity of R-Ras [14]. Work from the same lab further found that PLXND1 stimulated ARF6 GTPase activity by local production of phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) by type I phosphatidylinositol-4-phosphate-5-kinase (PIP5K) β [55]. Both of these pathways acted to modulate Integrin presentation on the HUVEC surface [56]. The role of Integrins has not been fully elucidated in AT [45, 57]. Further, the role of endothelial cell-localized Integrins on AT formation and growth appears essentially unstudied. However, based on the established mechanisms described above, we speculate that Integrin composition on the surface of VAT endothelial cells may play an important role in PlxnD1-mediated regulation of BFD.

To test this hypothesis it will be necessary to manipulate the Integrin code on vascular endothelial cells and assess effects on ECM and VAT growth. Such experiments may be conducted by using the *Tie2-Cre*^{Tg} (*Tek-Cre*) transgenic mouse line [58] to produce vascular endothelial cell-specific Integrin knockouts. Similar experiments have been performed previously to assess an endothelial cell-specific role for β1 Integrins [59-62]. As we hypothesize that β1 Integrins mediate crosstalk between VAT endothelial cells and the ECM to regulate VAT growth [55], it will be essential to temporally control Cre-mediated recombination due to embryonic defects in β1 Integrin knockout mice by using inducible Cre lines [59]. Although the conditional knockout strategy described above allows the ablation of Integrins to be restricted to endothelial cells, and further controlled by using inducible Cre lines, it would also be desirable to restrict Integrin ablation to endothelial cells specifically within VAT. However, to our knowledge no such transgenic line currently exists that expresses solely in VAT endothelial cells. Therefore, such an experimental strategy will induce Integrin knockout in endothelial cells across the body, potentially leading to secondary effects on VAT growth. Molecular profiles of tissue-specific endothelial cells has been performed for a variety of tissue types [63], therefore a similar strategy in adipose tissues may yield adipose-specific endothelial cell profiles that may be utilized for

transgenic strategies. However, to circumvent secondary effects, endothelial cell and adipocyte co-cultures may also need to be performed [64, 65].

Acknowledgements

This work was supported by American Heart Association Postdoctoral Fellowships (11POST7360004 and 13POST16930097), a University of Edinburgh and British Heart Foundation Fellowship, and a Diabetes UK grant (16/0005494) to JENM; and grants from NIH (R56-DK091356, R21-ES023369, R01-DK093399), and a Pilot Research Project Award from the University Cancer Research Fund at UNC Chapel Hill to JFR.

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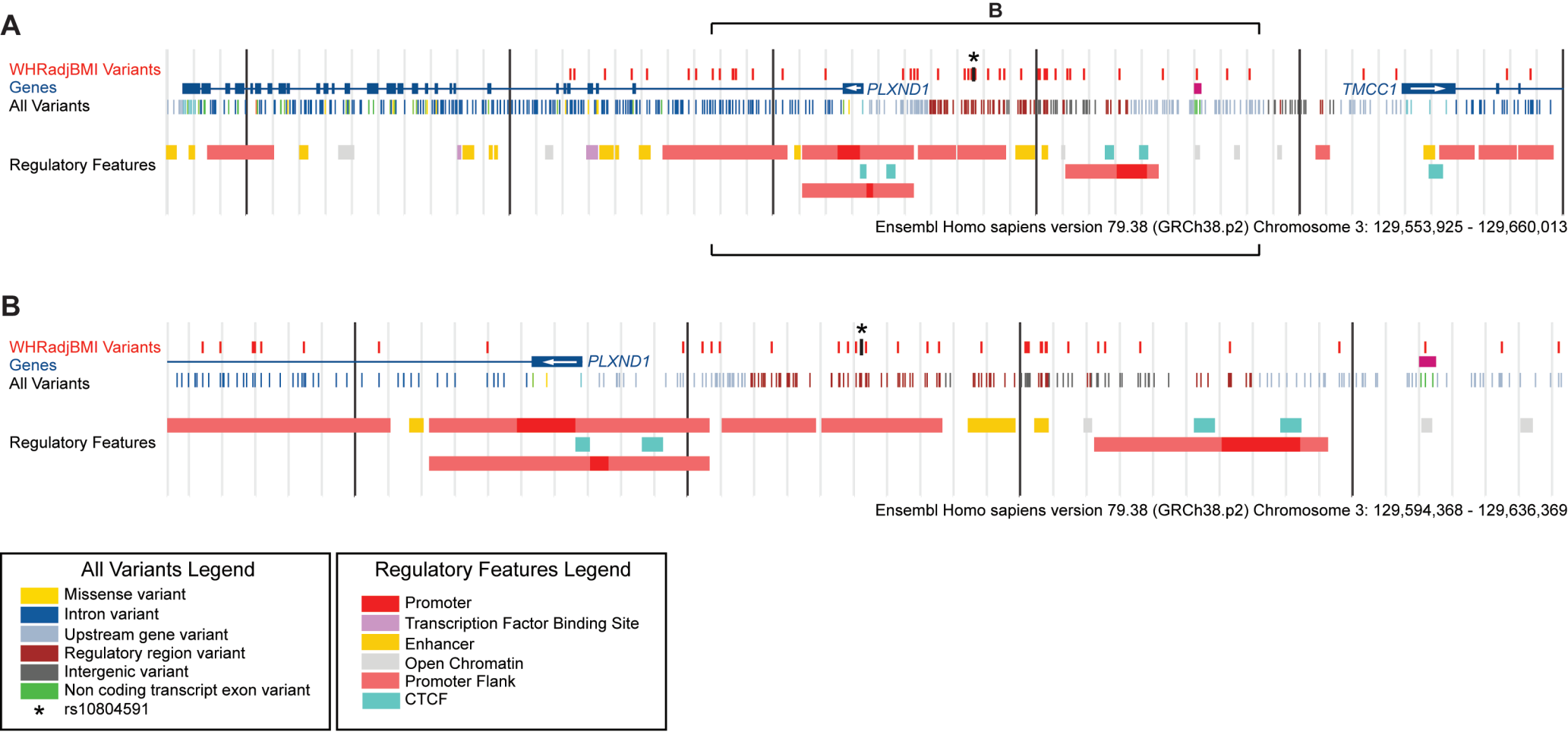
Figure Legends

Figure 1. Common variants and regulatory features at human *PLXND1*. Four tracks are depicted at the *PLXND1* locus. In descending order: WHRadjBMI Variants; 49 variants linked to rs10804591 (European ancestry, 1000G Phase 1, >0.7 R^2 in linkage disequilibrium of rs10804591). Genes; Havana annotated genes. Arrows within the first exon indicate direction of transcription. All Variants; common variants from 1000G Phase 1 with a frequency of at least 1% within populations of European ancestry. Regulatory Features; regulation marks predicted by the Ensembl Regulatory Build [66]. Vertical black bars are 100 kb (A) or 10kb (B) apart. Brackets in A denote the region shown in B.

Figure 2. β-coefficients and standard deviation for rs10804591. Bar charts indicate the β-coefficients and standard deviation (SD) for rs10804591 for waist-hip ratio adjusted for BMI (A; WHRadjBMI), waist circumference adjusted for BMI (WCadjBMI), and hip circumference adjusted for BMI (C, HIPadjBMI). All data are taken from Shungin et al. (2015). Asterisks indicate genome-wide significance ($P < 5x10\text{-}8$). Data are classified into 3 groups; sex-combined (black bars), female-only (white bars), and male-only (grey bars). Data are from GWAS or metabochip (MC) cohorts as described in Shungin et al. (2015).

Figure 3. Fluorescent lipophilic dyes to study body fat distribution in zebrafish. Nile Red stained zebrafish demonstrating neutral lipid stored within ATs (labelled in yellow) at two developmental stages. The arrows indicate VAT. SL = standard length (a measure of the fish length from the snout to the caudal peduncle).

Figure 4. Schematic illustrating the hypothesized mechanism by which vascular endothelial cell-derived Plxnd1 determines ECM composition and VAT expandability. A. Overview of the hyperproliferative and hyperplastic microenvironment of *plxnd1* mutant zebrafish VAT. **B.** Schematic on the interaction between PlxnD1, Integrins and ECM composition.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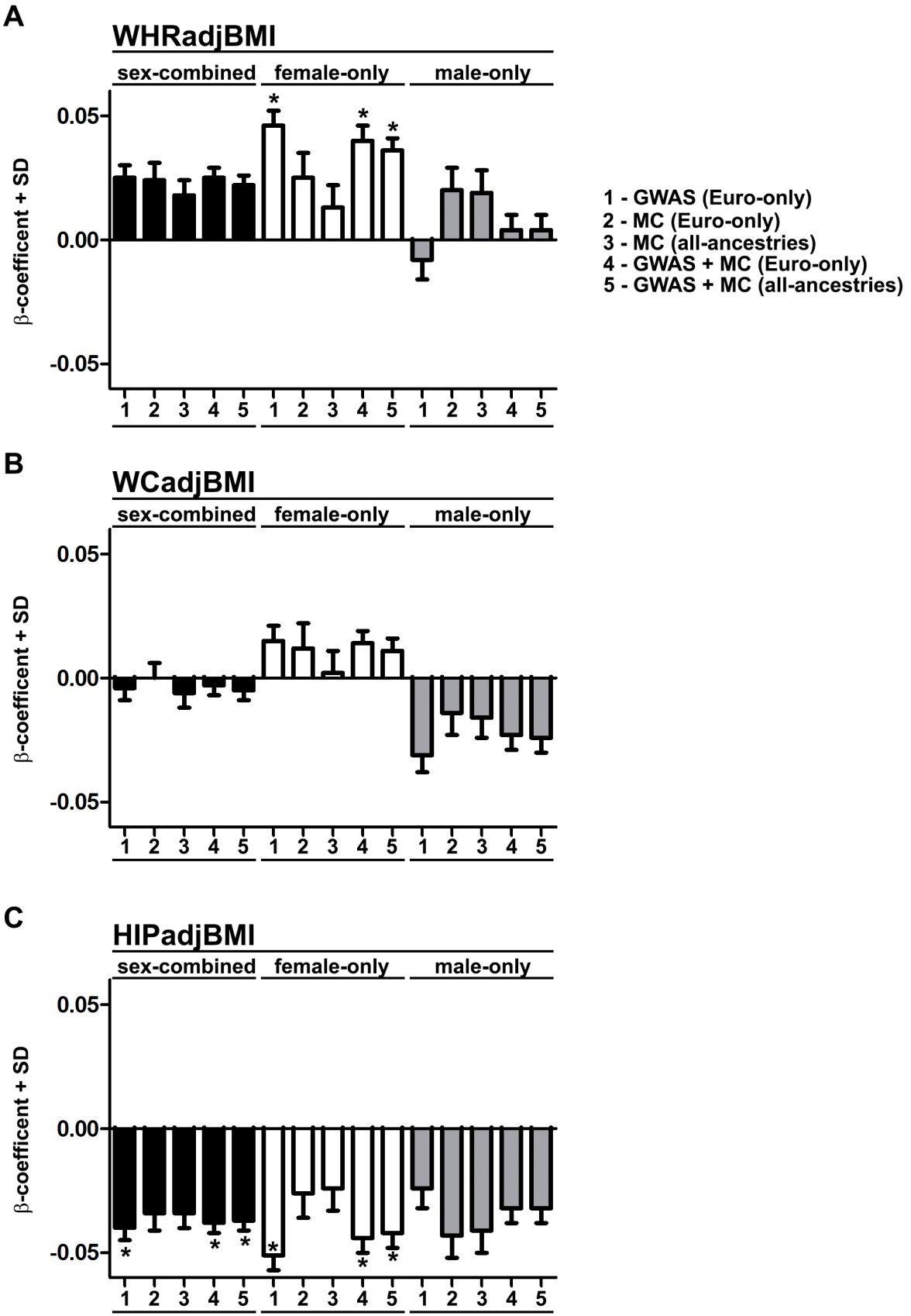
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3 - MC (all-ancestries)
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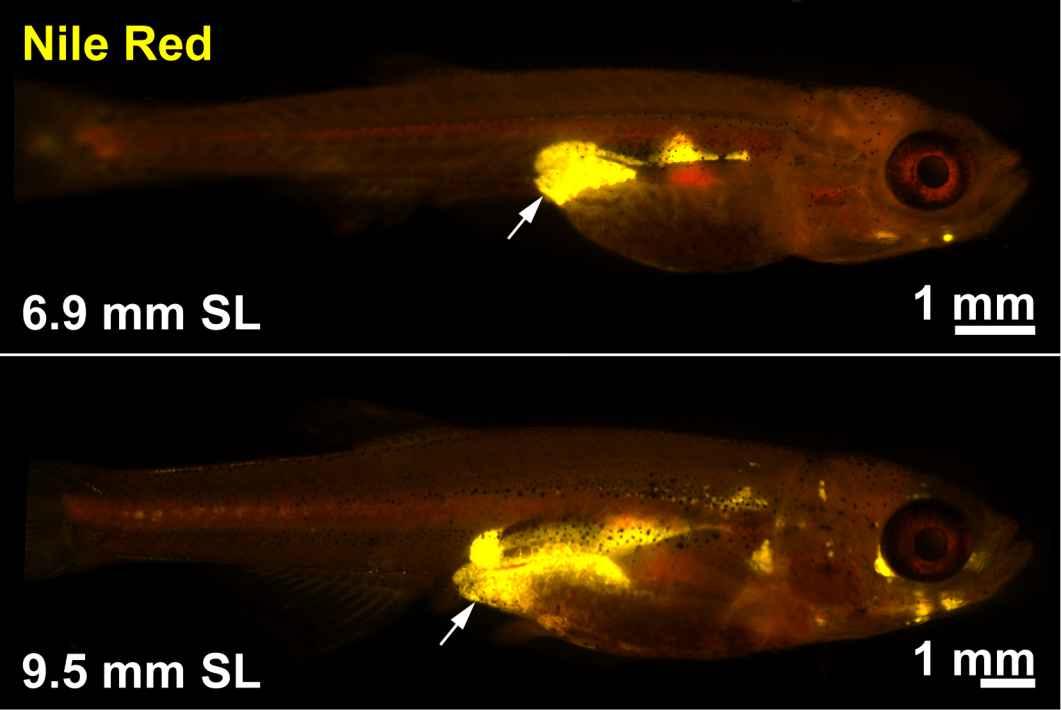
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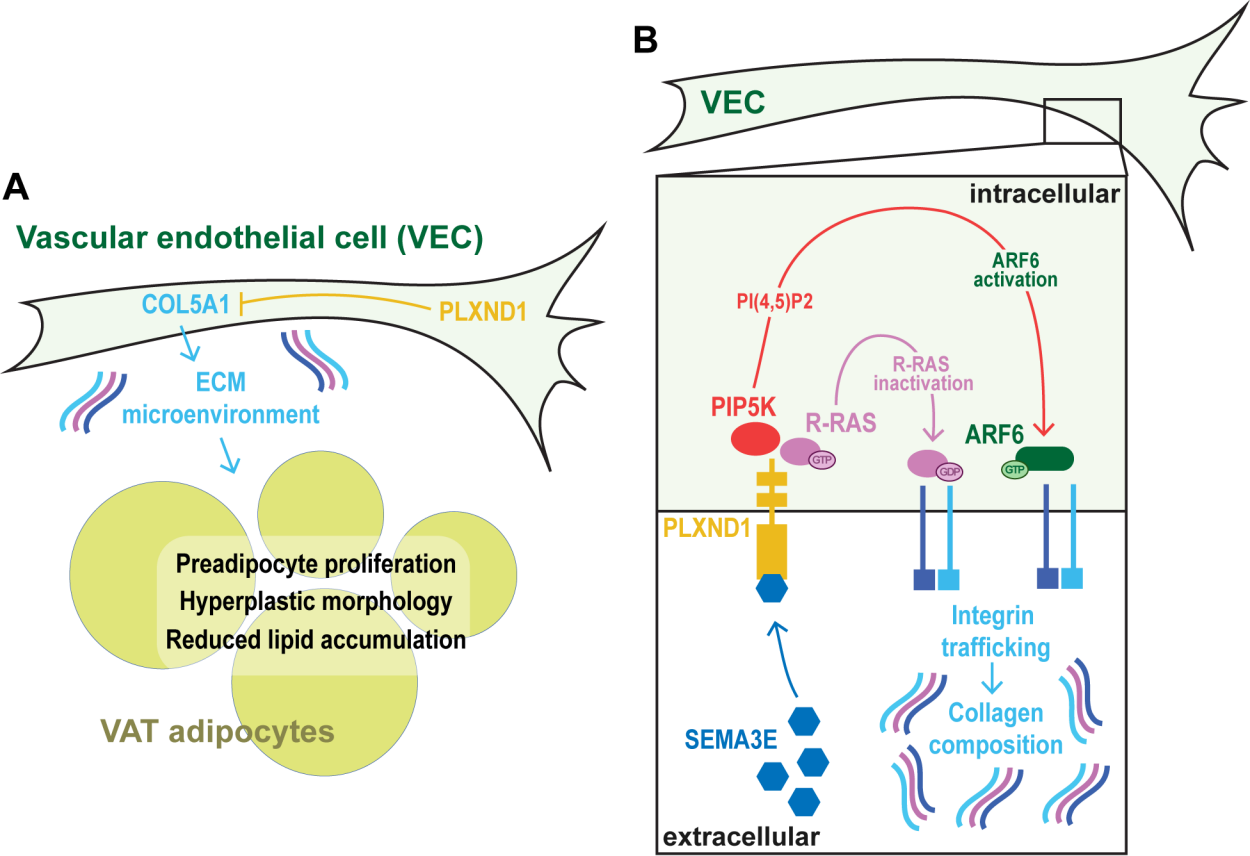
6.9 mm SL
1 mm
9.5 mm SL
1 mm


A
Vascular endothelial cell (VEC)
COL5A1
PLXND1
ECM
microenvironment
Preadipocyte proliferation
Hyperplastic morphology
Reduced lipid accumulation
VAT adipocytes
B
VEC
intracellular
PI(4,5)P2
ARF6
activation
R-RAS
inactivation
PIP5K
R-RAS
GTP
GDP
GTP
ARF6
PLXND1
Integrin
trafficking
Collagen
composition
SEMA3E
extracellular