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# Determination of obesity associated gene variants related to TMEM18 through ultra-deep targeted re-sequencing in a case-control cohort for pediatric obesity

MATHIAS RASK-ANDERSEN[1]*, MARKUS SÄLLMAN ALMÉN[1], JOSEFIN A. JACOBSSON[1], ADAM AMEUR[2], GEORGE MOSCHONIS[3], GEORGE DEDOUSSIS[3], CLAUDE MARCUS[4], ULF GYLLENSTEN[2], ROBERT FREDRIKSSON[1] AND HELGI B. SCHIÖTH[1]

[1]*Department of Neuroscience, Functional Pharmacology, Uppsala University, BMC, Uppsala, Sweden*
[2]*Department of Immunology, Genetics, and Pathology, SciLifeLab Uppsala, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden*
[3]*Department of Nutrition and Dietetics, Harokopio University of Athens, Athens, Greece*
[4]*Department for Clinical Science, Intervention and Technology, Karolinska Institutet, Division of Pediatrics, National Childhood Obesity Centre, Stockholm, Sweden*

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**Summary**

Genome-wide association studies (GWAS) have revealed association of a locus approximately 25b downstream of the TMEM18 gene with body mass and obesity. We utilized targeted re-sequencing of the body mass associated locus in proximity of TMEM18 in a case-control population of severely obese children and adolescents from the Stockholm area. We expanded our study to include the TMEM18 gene itself, with the aim of identifying body mass associated genetic variants. Sequencing was performed on the SOLiD platform, on long-range PCR fragments generated through targeted amplification of the regions of interest. Candidate single nucleotide polymorphisms (SNPs) were validated by TaqMan genotyping. We were able to observe 131 SNPs across the re-sequenced regions. Chi squared tests comparing the allele frequencies between cases and controls revealed 57 SNPs as candidates for association with obesity. Validation and replication genotyping revealed robust associations for SNPs within the haplotype block region located downstream from the TMEM18 gene. This study provides a high resolution map of the genetic variation pattern in the TMEM18 gene, as well as the associated haplotype block, and further strengthens the association of variants within the proximal haplotype block with obesity and body mass.

## 1. Introduction

Our understanding of the genetic components behind obesity has been greatly expanded in the last few years with the identification of new body mass associated genetic loci through GWAS (Thorleifsson *et al.*, 2009; Willer *et al.*, 2009; Heid *et al.*, 2010; Speliotes *et al.*, 2010). These large-scale studies have consistently identified the same common genetic variants to be strongly associated with BMI and obesity: e.g. variants within or in proximity to FTO, MC4R, TMEM18, SH2B1, KCTD15, BDNF, MTCH2, NEGR1, SEC16B, GNPDA2 and ETV5 (Thorleifsson *et al.*, 2009; Willer *et al.*, 2009; Heid *et al.*, 2010; Speliotes *et al.*, 2010). However, many of these genes have poorly understood functions and the mechanisms through which they influence obesity remain to be described, a process made more complicated by the fact the genes are expressed in numerous tissues and thus involved in multiple biological pathways. Nevertheless several of the obesity-associated genes such as FTO, MC4R and TMEM18 show high levels of expression in the hypothalamus, a region of the brain known to regulate food intake and energy balance (Schwartz & Porte, 2005; Berthoud *et al.*, 2006; Bauer *et al.*, 2009).

The TMEM18 gene is located at chromosome 2p25·3, approximately 670 kbp from the end of the short arm of chromosome 2. TMEM18 encodes the transmembrane protein 18, a poorly characterized protein in terms of function. The expression profile of TMEM18 conducted in conjunction with the GWAS performed by the GIANT consortium (Willer *et al.*, 2009) indicated that it is expressed

* Corresponding author: E-mail: mathias.rask-andersen@neuro.uu.se

ubiquitously, although not with an identical pattern in all tissues. The expression of TMEM18 in the brain of humans and mice (Almen *et al.*, 2010), particularly in the hypothalamus, has pointed to a function in regulation of homeostasis. Moreover, correlation between prefrontal cortex genetic expression of TMEM18 with body weight in rats has suggested a potential role for TMEM18 in higher cognitive functions related to feeding behavior (Rask-Andersen *et al.*, 2012). Functional studies by Jurvansuu and colleagues have revealed TMEM18 to localize to the nuclear envelope of neural stem cells (Jurvansuu *et al.*, 2008), and to be involved in cell migration. More recently the same group identified TMEM18 as a nuclear membrane protein that sequesters DNA along the nuclear membrane, simultaneously suppressing transcription (Jurvansuu & Goldman, 2011).

Our group has previously investigated the rs6548238 and rs4854342 SNPs and reported their association with obesity and body mass in a case-control cohort of obese children from the Stockholm area, as well as in a cross sectional cohort of Greek children and adolescents (Almen *et al.*, 2010; Rask-Andersen *et al.*, 2012). In this project, we utilized the SOLiD sequencing platform for targeted re-sequencing of the TMEM18 downstream haplotype block containing the body mass associated genetic locus, as well as the TMEM18 gene itself, in pooled samples of DNA, representing the entire case-control cohort of 524 severely obese children and 527 controls of Swedish ancestry from the Stockholm area, with the purpose of mapping genetic variation in the region and identifying novel SNPs that may be associated with obesity. Candidate SNPs associated with obesity by comparing allele frequencies were then validated by TaqMan genotyping and replicated in the cross sectional cohort of Greek children and adolescents.

## 2. Materials and methods

### (i) *Obesity case-control cohort*

Re-sequencing of the TMEM18 gene was conducted on a population comprising severely obese subjects enrolled at the pediatric unit of Karolinska Hospital in Huddinge, Sweden and a control group from the same region, previously described (Jacobsson *et al.*, 2008; Sallman Almen *et al.*, 2013). The severely obese group was composed of 524 children (270 girls and 254 boys with mean age of $12{\cdot}7 \pm 3{\cdot}2$ years and BMI z-score of $3{\cdot}5 \pm 0{\cdot}06$) registered at the National Childhood Obesity Centre, Karolinska University Hospital, Huddinge. The group of control subjects consisted of 527 healthy and normal weight Swedish adolescents (271 girls and 256 boys with a mean age of $17{\cdot}0 \pm 0{\cdot}9$ years and a BMI z-score of $0{\cdot}06 \pm 0{\cdot}8$) recruited from 17 upper secondary schools in the Stockholm area. We conducted our investigation with the approval of the Regional Committee of Ethics, Stockholm, with participants and their guardians participating willingly and having provided their informed consent in writing. Genomic DNA was extracted from peripheral blood cells using a QiaGen Maxiprep kit (Qiagen, Hilden, Germany). DNA concentrations were measured with a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Delaware, USA).

### (ii) *Re-sequencing: primer design and DNA amplification*

Equal amounts of DNA from each individual were combined into pools of 170–190 samples, resulting in a total of three DNA pools of obese and control individuals. PCR primers were designed employing Primer Premier 5·0 and Primer 3Plus software (Premier Biosoft, California, USA) (Untergasser *et al.*, 2007) with a target product size of approximately 1500–2200p. A total of 28 overlapping primers were designed to cover the haplotype block observed to be associated with body mass in previous GWAS (Willer *et al.*, 2009), as well as TMEM18 gene itself.

For each primer pair, PCR amplification (UnoCycler, VWR, Sweden) was applied to each pool in duplicates. DNA samples at 50g were diluted in a solution composed of 0·3μM primer, 5x buffer containing 2·0M $MgCl_2$ (Kapa Biosystems, Massachusetts, USA), 0·3M dNTP (Kapa Biosystems, Massachusetts, USA) and 0·02/μl KAPA HiFi DNA polymerase (Kapa Biosystems, Massachusetts, USA) to a total volume of 25μl. The annealing temperature was optimized for each individual primer pair. During the amplification process samples were first submitted to an initial denaturation at 95 °C for 2 minutes, followed by 50 cycles of denaturation at 98 °C for 30, annealing at optimal temperature for 15 and extension at 72 °C for 80. Correct sizes of PCR products were verified by gel electrophoresis. DNA was extracted from the gel with the GeneJET Extraction Kit (Fermentas Life Sciences, Helsingborg, Sweden). Amplicon concentrations were then measured in triplicate using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Delaware, USA). Amplicons were combined in equal amounts, compensating for amplicon length, into two pools, obese children and normal weight controls, to a total amount of 2·5μg DNA.

### (iii) *SOLiD sequencing and analysis*

The sequencing libraries from each pool were prepared following the instructions from Applied Biosystems, as was the emulsion-PCR. Re-sequencing was executed on the Applied Biosystems' SOLiD 3 platform using 50p read length on standard slides as specified by the manufacturer's protocol. Applying the Bioscope v1.3

algorithm (Applied Biosystems, Foster City, USA) the sequence reads were mapped to the TMEM18 reference sequence (hg19, chr2:667 974–677 438). The coverage was calculated for each site across the targeted region by counting the number of overlapping reads.

SNPs were identified as valid adjacent mis-matches in the colour space sequence as compared to the reference sequence. The number of uniquely placed sequence reads for each position was also calculated as described previously (Zaboli *et al.*, 2012). Qualitative measurements of sequence depth and number of unique reads, 'unique valid adjacent mis-match' (UVAM)-score, were calculated for each SNP and used to filter out variations with low coverage. The threshold for filtering out false-positive SNPs based on UVAM scores was obtained from comparing the distribution among known SNPs present in the dbSNP database (v133, http://www.ncbi.nlm.nih.gov/projects/SNP/) and the 1000 Genome Project (http://www.1000genomes.org/) to the distribution among novel SNP candidates in the sequenced region. The considerable difference in distribution was interpreted as a consequence of a large proportion of novel candidates not being truly polymorphic. Under this assumption, a binomial approximation of the hypergeometric distribution was used to model the probability of false SNPs to have a specific UVAM score. Using this model, the UVAM threshold for a false-discovery rate (FDR) of 1% was calculated to be a score of 21 and 23 for obese and control pools, respectively. Consequently, candidate SNPs with scores equal to or below these thresholds were filtered out and not included for further analyses.

For our analysis, we used a cut-off of at least 23% coverage on each DNA strand. Candidate SNPs also had to be detected in both the obese and control population. The filtering approach described above was tested against a method that employed a minimum of assumptions for SNP calling and based on the SOLiD system's valid adjacent mis-matches approach as described in Sallman Almen *et al.* (2013). As in the previous method, candidate SNPs in the primer regions, with total coverage of less than 500 reads and not present in both groups were excluded from further analysis. A total of 131 SNPs could be observed in our dataset after applying the aforementioned filters; a total of 14 within the TMEM18 gene and 117 in the proximal haplotype block. Association of candidate SNPs with obesity was tested for using Chi squared tests. Multiple testing was corrected for using FDR. Out of 131 SNPs, 57 were observed to be associated with obesity when applying FDR (Supplementary Table 1).

### (iv) *Validation genotyping*

TaqMan genotyping was performed in the Swedish case-control cohort. We also had access to a well characterized cross sectional cohort of Greek children and adolescents with ages ranging from 9 to 13 years old. This cohort is part of the 'Healthy Growth Study', a large-scale cross-sectional epidemiological study initiated in May 2007 and described previously (Moschonis *et al.*, 2010). Swedish and Greek populations, despite having a common origin, display differences in genetic variation; however, the effects of TMEM18 proximal genetic variants on body mass have been observed in several populations of unique origins, providing a good chance of replicating or validating results between the Swedish and Greek cohorts of children and adolescents. DNA was available for 2352 subjects for genotyping (1311 girls and 1064 boys with BMI z-score of $20{\cdot}3 \pm 0{\cdot}1$). Each child participated willingly and guardians provided their informed consent in writing for the study, approval was also granted by the Greek Ministry of National Education and the Ethical Committee of Harokopio University of Athens.

Genotyping was performed by means of pre-designed Taqman SNP genotyping assays (Applied Biosystems, Foster City, USA) on an ABI7900 genetic analyzer with SDS 2·2 software. This was conducted on both the Swedish and Greek cohorts described above. Data filtering and association tests for each SNP were performed in the R program package (R Development Core Team, 2010) with locally developed scripts. The results obtained from the individual genotyping was analyzed with the PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/). Deviation from Hardy-Weinberg equilibrium was tested for each validated SNP using a Pearson's Chi squared test with 1 degree of freedom. Logarithmic regression was utilized to test for association of SNPs with obesity in the cohort of Swedish children and adolescents. Models were adjusted for gender and assumed an additive effect. Linear regression was utilized to test for association of SNPs with BMI z-score in the cohort of Greek children and adolescents. Models were adjusted for gender, age and stage of pubertal development (Tanner stage). Linkage disequilibrium (LD) analysis was also performed in PLINK.

### (v) *Visualization of data*

Results obtained from both sequencing and statistical analysis were graphically visualized and integrated with data from the ENCODE Project (February 2012), the NHGRI catalogue of published GWAS (February 2012) and dbSNP (version 135) using the UCSC genome browser (http://genome.ucsc.edu/). Information regarding regulation of transcription from the ENCODE project was integrated, more specifically: data on histone modification (overlayed H3K4Me1, H3K27Ac and H3K4Me3) from the Bernstein lab at the Broad Institute, DNaseI hypersensitivity clusters and transcription factor binding

site information generated from ChIP-seq provided by the Myers lab at the HudsonAlpha Institute for Biotechnology and the labs of Michael Snyder, Mark Gerstein and Sherman Weissman at Yale University, Peggy Farnham at UC Davis and Kevin Struhl at Harvard (Moskal *et al.*, 2011). All genome positions used in this study refer to the February 2009 assembly (NCBI37/hg19).

## 3. Results

### (i) *SOLiD sequencing*

Sequencing was performed on amplicons of the region containing the entire human TMEM18 gene and the body mass associated downstream haplotype block. A total of 69 290 bases were successfully sequenced (Fig. 1); the non-coding region between the gene and the haplotype block was excluded from sequencing. Average coverage was determined by calculating the number of overlapping reads for each site and was 30 059 and 66 263 in the obese and control pools, respectively. Additionally, approximately 93% of all positions had a minimum coverage of 1000 in both pools. Although the two independently sequenced pools possessed a very similar pattern of coverage peaks for each position, the coverage itself was slightly more than doubled in the control pool of the haplotype region (Fig. 1).

Primer regions were excluded from our analysis. Regions in direct proximity of primer sequences exhibited abnormally high coverage (up to 386 200 and 814 530 reads in the obese and control population, respectively). Our previous study utilizing the SOLiD platform (Sallman Almen *et al.*, 2013) found SNP calling to be prone to detect false positives in locations where PCR amplicons overlapped. These regions were consequently excluded from further analysis, leaving a total of 41 820 bases for calling.

SNP calling was performed by the SOLiD system, which identified candidate SNPs from reads containing valid adjacent mis-matches. To remove potential false-positive SNPs we applied the statistical approach described above. A total of 131 SNPs were found to fulfill the criteria and thus considered viable candidates for association tests. Chi squared tests for associations with obesity were performed for each candidate SNP. p-values were adjusted for by controlling the FDR as proposed by Benjamini and Hochberg (1995). Applying this method, an unadjusted p-value less than 0·023 was considered significant. A total of 57 SNPs, two in intron 3 of the TMEM18 gene and 55 within the proximal haplotype block, were observed to be associated with obesity. (Fig. 1).

We selected four SNPs that we observed to be associated with obesity in the re-sequencing data for validation and replication on the TaqMan genotyping platform. Three SNPs were selected that were approximately evenly distributed across the body mass associated locus located downstream from TMEM18: rs17042114 at position 615 819 (human genome assembly GRch38), rs11274848 at 623 798 and rs4854342 at 634 476; and one SNP from within the TMEM18 gene: rs10168969 at position 672 496 (Fig. 1). We also included in our analysis genotyping data for rs6548238 and rs4854344 from previous studies by this group (Almen *et al.*, 2010; Rask-Andersen *et al.*, 2012), and expanded genotyping of these variants to include children and adolescents in the Greek cohort. Variation at rs6548238 could be observed in our SOLiD data; however, this position did not fulfill the criteria for coverage set in our analysis and was thus excluded from further analysis.

### (ii) *Replication and validation of candidate SNPs through TaqMan genotyping*

Results from validation and replication genotyping of candidate SNPs are presented in Tables 1 and 2, and visualized in Fig. 1. rs1127484, rs6548238 and rs4854344 were observed to be associated with obesity in the case-control cohort of Swedish children and adolescents at the adjusted threshold for significance ($p < 0{\cdot}023$). rs10168696 could be observed to be associated with obesity at the nominal level of significance; however, it did not reach the adjusted threshold of significance taking into account multiple testing. In the cross sectional cohort of Greek children and adolescents, linear regression revealed associations of rs6548238 and rs4854344 with BMI z-score at the adjusted threshold for significance ($p < 0{\cdot}023$). The association of rs11127484 did not reach the adjusted level of significance for association with BMI z-score, despite association at the nominal level of significance. Variation of rs4854342 was not observed in either cohort, which indicates the result at this position in the SOLiD resequencing to be a false positive. LD analysis revealed three SNPs within the TMEM18 downstream region haplotype block to be in LD: rs11127484, rs6548238 and rs4854344 ($r^2 > 0{\cdot}90$). No linkage between downstream SNPs and SNPs within intron 3 of TMEM18 could be observed in our dataset.

## 4. Discussion

We generated a high resolution map of the genetic variation patterns associated with obesity in the TMEM18 gene and associated haplotype block. Having, in total, sequenced approximately 69 kbp with very high coverage we were able to identify 57 candidate SNPs: 55 within the proximal haplotype block and two within intron 3 of TMEM18. We were able to validate the association of rs11127484,

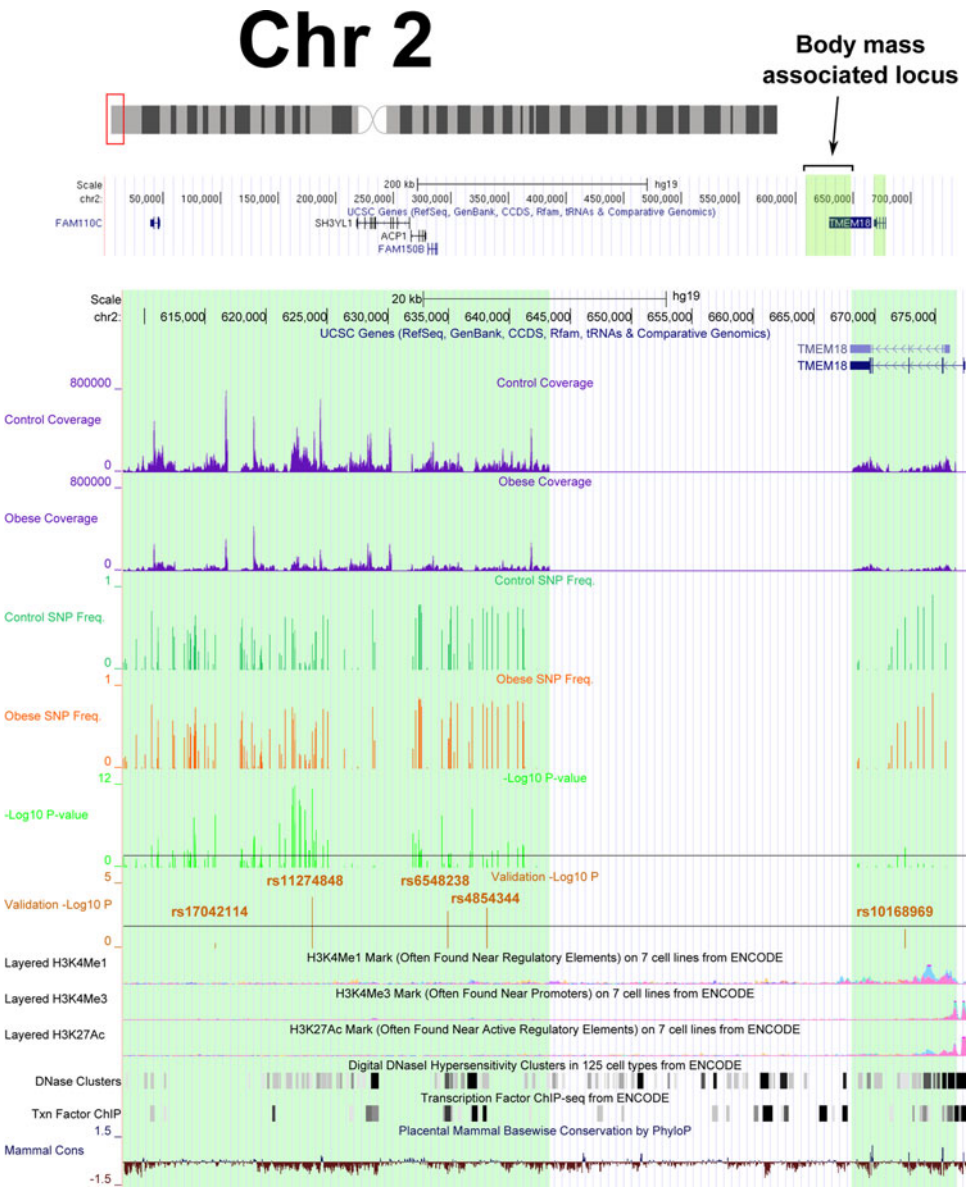


Fig. 1. Graphical representation of the location of TMEM18 on chromosome 2p25·3. Resequenced regions are highlighted in green. Coverage was measured as the number of reads mapped to each site achieved during re-sequencing of the obese and control DNA pools. Allelic frequencies of the alternative (non-reference allele) for all genetic variants detected during the re-sequencing in the control (green) and obese (orange) DNA pools are shown. Associations with obesity and odds ratios were determined by applying the Chi squared test. Negative log10 p-values are displayed. 57 SNPs were identified after adjusting for multiple testing with the FDR. p-values < 0·023 were considered significant, represented here by the black line. A total of 55 SNPs present in the TMEM18-linked haplotype block, were identified as candidate SNPs for association with obesity. Additional information was gathered from the ENCODE project and integrated with the sequencing data. Markers for functional genomic elements included modifications of histone 3: mono-methylation of lysine 4 (H3K4Me1), tri-methylation of lysine 4 (H3K4Me3), as well as acetylation of lysine 27 (H3K27Ac); indicating transcription enhancer sites. Different cell lines are represented in different colours (additional information can be obtained via the UCSC website at http://genome.ucsc.edu/). DNAse hypersensitivity regions, usually related to accessible chromatin, were included. Also included are transcription factor binding sites obtained from ChIP-seq. Darker colouration corresponds to stronger signal for possible binding sites. Conservation in vertebrate species for each base pair in the genomic sequence is included (Mammal Cons).

rs6548238 and rs4854344 with obesity in the case-control cohort of Swedish children. Replication in the cross sectional cohort of Greek children and adolescents revealed rs6548238 and rs4854344 to be associated with body mass at the adjusted level of significance. Borderline significant effects could also be observed for rs11127484 ($p = 0{\cdot}043$). The association of rs17042114 with obesity observed in our re-sequencing data was not observed when this SNP was genotyped with the TaqMan assay (Tables 1

Table 1. *Logistic regression analysis of candidate SNPs for association with obesity in the case-control cohort of Swedish children and adolescents.*

| SNP | Position | Region | Genotype Control Obese | OR (95% CI) | p-value |
|---|---|---|---|---|---|
| rs17042114 | Chr 2:615 819 | Downstream | AA/AG/GG<br>22/174/319<br>25/157/273 | 1·10 (088–1·36) | 0·40 |
| rs11127484 | Chr 2:623 798 | Downstream | TT/TC/CC<br>20/154/341<br>6/99/350 | 0·60 (0·47–0·78) | 1·11E-04 |
| rs6548238 | Chr 2:634 905 | Downstream | TT/TC/CC<br>19/148/353<br>7/102/357 | 0·66 (0·51–0·85) | 1·33E-03 |
| rs4854344 | Chr 2:638 144 | Downstream | GG/GT/TT<br>19/146/342<br>7/101/356G | 0·65 (0·50–0·83) | 7·79E-04 |
| rs10168696 | Chr 2:672 496 | TMEM18 intron 3 | TT/TC/CC<br>13/118/384<br>14/130/313 | 1·30 (1·02–1·66) | 0·035 |

Models were corrected for gender.

and 2), despite our efforts to filter poor quality SNPs based on coverage. This candidate SNP is thus a clear false positive generated by the resequencing methodology. It should be mentioned that rs6548238 was filtered out by our approach, which may indicate more lenient criteria for coverage to be suitable.

Two candidate SNPs for association with obesity were identified within the TMEM18 gene through re-sequencing. rs10168696, despite showing significance at the nominal level, did not reach statistical significance in our validation in the cohort of Swedish children and adolescents. Association of this SNP to body mass could also not be observed in the Greek cross sectional cohort. Our observation may be indicative of effects of rs10168696 on body mass that are specific for the Swedish population; however, given the nominal level of significance of this association, we cannot rule out this as a false-positive observation.

The results from our validation genotyping are in line with observations reported by previous GWAS that reported strong signals within a proximal downstream haplotype that decline outside this region (Willer *et al.*, 2009). Putative regulatory genetic elements from the ENCODE database are visualized in Fig. 1. A dense region of indicators of regulatory elements: DNase hypersensitivity clusters, histone modifications and transcription factor binding sites can be observed in the immediate TMEM18 upstream region, as is common in gene promoter sites. Regulatory elements can also be observed within the haplotype block region, particularly near rs6548238 and rs4854344, which may indicate potential causal variants in the vicinity of these sites.

In conclusion, we utilized the SOLiD sequencing platform for targeted re-sequencing of the TMEM18 gene and downstream haplotype region in pooled DNA samples of a case-control cohort of obese

Table 2. *Linear regression analysis of candidate SNPs for association with BMI z-score in the cohort of Greek children.*

| SNP | Position | Region | Minor allele | B-value | p-value |
|---|---|---|---|---|---|
| rs17042114 | Chr 2:615 819 | Downstream | AA/AG/GG<br>32/620/1691 | 0·05 | 0·32 |
| rs11127484 | Chr 2:623 798 | Downstream | TT/TC/CC<br>95/758/1490 | −0·09 | 0·043 |
| rs6548238 | Chr 2:634 905 | Downstream | TT/TC/CC<br>91/751/1499 | −0·10 | 0·023 |
| rs4854344 | Chr 2:638 144 | Downstream | GG/GT/TT<br>92/748/1501 | −0·11 | 0·017 |
| rs10168696 | Chr 2:672 496 | TMEM18 intron 3 | TT/TC/CC<br>30/520/1793 | −0·09 | 0·099 |

Models were corrected for gender, age and stage of pubertal development (Tanner stage)

children. This is a cost-effective approach that allowed fine-mapping of genetic variation at the TMEM18 locus as well as the study of potential population-specific effects and the possibility to detect new rare causative genetic variants. Using this approach, we were able to observe 131 SNPs across the re-sequenced regions. Chi squared tests comparing the allele frequencies between cases and controls revealed 57 SNPs to be candidates for association with obesity. In comparison, nine SNPs at this locus were reported to be associated with BMI by the GIANT consortium (Speliotes *et al.*, 2010). Validation and replication genotyping revealed robust associations for SNPs within the haplotype block region located downstream from TMEM18, in line with results seen in previous studies (Hotta *et al.*, 2009; Willer *et al.*, 2009). SNPs within the TMEM18 gene were not validated by TaqMan genotyping. This indicates the effects of genetic variation at the TMEM18 locus to be more closely related to genomic functionality within the downstream haplotype block rather than to genetic variants within the TMEM18 gene itself.

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## Declaration of interest

None.

## Supplementary material

The online supplementary material can be found available at http://journals.cambridge.org/GRH

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