Global mapping transcriptional start sites revealed both transcriptional and post-transcriptional regulation of cold adaptation in the methanogenic archaeon *Methanolobus psychrophilus*

Jie Li1*, Lei Qi1*, Yang Guo2, Lei Yue1, Yanping Li2, Weizhen Ge2, Jun Wu2, Wenyan Shi3 & Xiuzhu Dong1

1State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, No.1 Beichen West Road, Beijing 100101, People’s Republic of China, 2Novogene Bioinformatics Institute, 21st Floor, Jinma building B area, Xueqing Road, Beijing 100083, People’s Republic of China, 3Department of Microbiology, Immunology and Molecular Genetics, University of California, 10833 Le Conte Avenue, Los Angeles, CA90095, USA.

Psychrophilic methanogenic Archaea contribute significantly to global methane emissions, but archaeal cold adaptation mechanisms remain poorly understood. Hinted by that mRNA architecture determined secondary structure respond to cold more promptly than proteins, differential RNA-seq was used in this work to examine the genome-wide transcription start sites (TSSs) of the psychrophilic methanogen *Methanolobus psychrophilus* R15 and its response to cold. Unlike most prokaryotic mRNAs with short 5′ untranslated regions (5′ UTR, median lengths of 20–40 nt), 51% mRNAs of this methanogen have large 5′ UTR (>50 nt). For 24% of the mRNAs, the 5′ UTR is >150 nt. This implies that post-transcriptional regulation may be significance in the psychrophile. Remarkably, 219 (14%) genes possessed multiple gene TSSs (gTSSs), and 84 genes exhibited temperature-regulated gTSS selection to express alternative 5′ UTR. Primer extension studies confirmed the temperature-dependent TSS selection and a stem-loop masking of ribosome binding sites was predicted from the longer 5′ UTRs, suggesting alternative 5′ UTRs-mediated translation regulation in the cold adaptation as well. In addition, 195 small RNAs (sRNAs) were detected, and Northern blots confirmed that many sRNAs were induced by cold. Thus, this study revealed an integrated transcriptional and post-transcriptional regulation for cold adaptation in a psychrophilic methanogen.

Cold-adaptive methanogenic Archaea (methanoarchaea) contribute significantly not only to methane emissions from the wetlands in cold regions of the Earth, but also to the low-temperature biogas fermentations1. However, the mechanisms of cold adaptation used by these psychrophilic Archaea have not been well studied except for the Antarctic *Methanococcoides burtonii*2–4, probably because only a few cold-adaptive Archaea have been cultured. In this study, a domesticated psychrophilic methanogen *Methanolobus psychrophilus* R15 isolated from the cold Zoige wetland on Tibetan Plateau5, was used to explore archaeal cold adaptation mechanisms. Previous transcriptomic analysis of R15 indicated that expression of 40% of its genes responded to changes in temperature and that the genes specifically related to RNA degradation were up-regulated in response to cold6, implying that mRNA turnover may play a role in the cold-responsive gene regulation.

Cold has various impacts on biological systems, such as increasing the rigidity of the cell membrane and stabilizing mRNA secondary structure. The latter affects mRNA turnover and translation7. Because mRNA secondary structure responds to cold more promptly than proteins, changes in mRNA secondary structure may be a preferred mode in cold-responsive regulation. For example, the cold shock protein gene *cspA* of *E. coli* encodes an RNA chaperone and transcribes a large 5′ untranslated region (UTR) of mRNA which determines the transcript’s cold stability and translational conformation in the cold8. This unique architecture of the *cspA* mRNA allows it to function as a RNA thermometer9. Our recent work demonstrated that the mRNAs for *mtaA* and *mtaC*, which encode the methylcobalamin:coenzyme M methyltransferase and methanol corrinoid proteins that are the
key enzymes in methanol-derived methanogenesis of a cold-adaptive methanogen, all have long 5’ UTRs, that contribute to the transcripts’ stability at low temperatures25,26.

Precise transcript architecture can reveal diverse cis-RNA elements, including the 5’ and 3’ UTR and non-coding RNAs, those contribute significantly to gene regulation in Eukaryotes11,12. However, whole-transcript RNA sequencing often fails to identify the precise transcriptional architectures due to under-representation of the 5’ and 3’ ends of transcripts. By using differential RNA-seq (dRNA-seq), an approach that discriminates the primary (5’ triphosphorylated, 5’ PPP) from the processed (5’ mono-phosphorylated, 5’ P) transcripts, Jäger et al. have identified the genome-wide transcription start sites (TSSs) for *Methanosarcina mazei* Gö1. They found that the majority of the mRNAs possess long 5’ UTRs and discovered more than two hundreds of sRNAs13, suggesting the importance of post-transcriptional regulation in methanoarchaea. Following that study, this newly developed dRNA-seq approach has also been used in human pathogens *Helicobacter pylori*14, *Enterococcus faecalis*5, and cyanobacteria *Synechocystis*. These studies have identified genome-wide TSSs and revealed the response of the primary transcriptomes of bacteria to the environment.

Based on the hypothesis that mRNA structure-dependent post-transcriptional regulation could play a role in cold-adaptive methanogens, this work was designed to reveal the detailed changes of the genome-wide transcriptional architecture of *M. psychrophilus* R15 in response to cold. dRNA-seq was used to generate a temperature-dependent genome-wide TSS atlas of *M. psychrophilus* R15. The TSSs were further refined by assembly of the 5’-end libraries with the whole-transcript libraries, and some TSSs were experimentally confirmed. Extensive analyses were performed to understand the dynamic transcriptome in connection with the cold-response of this psychrophilic archaean.

**Methods**

**Strain cultivation and RNA extraction**. *M. psychrophilus* R15 was grown at 8 and 18°C in a mineral medium containing 20 mM trimethylamine under gas phase of 80:20 N2:CO2 as described7. Cells were harvested from the mid-log phase culture at 4°C, and total RNA was extracted using TRIzol. Briefly, frozen pellets collected from 20 mL of culture were lysed in 1 mL of TRIzol Reagent (Ambion). After 5 min of incubation at room temperature, 200 μL of chloroform was added. The mixture was shaken vigorously and centrifuged at 12,000 g for 15 min. The supernatant was combined with 500 μL of isopropanol and centrifuged at 12,000 g for 10 min. The pellet was washed with 70% ethanol, air-dried, and dissolved in RNase-free water.

**rRNA depletion**. rRNA was depleted at a high efficiency (Supplementary Table S1) using Ribo-ZeroTM rRNA Removal Kit (Epitope). To discriminate the primary transcripts from those with processed 5’ ends, two dDNA libraries were constructed as follows: a (+) library was enriched in primary transcripts (5’ triphosphorylated, 5’ PPP) by using the Terminator® 5’-polyphosphate-dependent exonuclease (TXP, Epitope) to deplete the processed RNAs (5’ mono-phosphohated, 5’ P). A (−) library without TEX treatment that contained both 5’ PPP- and 5’ P-transcripts was also prepared. Both the TEX-treated and untreated RNA samples were then subjected to a treatment of tobacco acid pyrophosphatase (TAP, Epitope) to generate 5’-P-containing linker ligation. Next, using an RNA-Seq Library Preparation Kit for Transcriptome Discovery (Gnomegen), dRNA-seq RNA libraries were constructed in accordance with the manufacturer’s recommendations. The cDNAs were size-fractioned within the range of 300 to 600 nt on agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen). In addition, for TSS refinement and verification, standard strand-specific whole transcript sequencing cDNA libraries (w library) were constructed for the total RNAs using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs) according to the manufacturer’s instructions.

High-throughput sequencing and quality control (QC). cDNA libraries were sequenced on an Illumina HiSeq 2000 platform. The short reads sequenced from one end of the cDNA fragments (single-end sequencing) was used for dRNA-seq libraries, but paired-end sequencing was used for the whole-transcript libraries; both produced reads of 100 bp in length. Images generated by a sequencing machine were converted to raw sequencing data using base calling (CASAVA version 1.8.2). Adapter sequences were trimmed from the reads and then subjected to the following pre-processed QC steps to discard all of the reads that meet any of the following thresholds: truncated reads of ≤1/3 of the original reads in length; reads with ≥10% uncalled bases (Ns); and reads with ≥50% of low-quality bases (PHRED quality scores ≤5).

**Read mapping and gene quantification**. QC filtered reads were aligned to the *M. psychrophilus* R15 reference genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/*Methanobacterium_...</now>
transcript sequencing. Three cDNA libraries, two different RNA sequencing approaches were combined: dRNA-cold response of the Genome-wide TSS and operon maps

Results

The reaction was terminated by incubation at 70°C for 15 min. Then 50 μL RNase reaction mix (100 mg/mL salmon sperm DNA, 20 μg/mL RNase A) was added. After incubation at 37°C for 15 min, the reaction products were extracted with phenol and chloroform and precipitated with ethanol and glycogen overnight at −80°C. DNA sequencing reactions were performed with the same [γ-32P] GTP-labelled primer by using a Sequi Thermo EXCEL II DNA sequencing system (Epigenetica) according to the manufacturer’s instructions. The products of the primer extension and DNA sequencing reactions were resuspended separately in the same sequencing loading buffer, denatured at 90°C for 5 min, and run on a 6% acrylamide sequencing gel. After electrophoresis, the gels were subjected to autoradiography on X-ray film.

Northern blot analysis. 2–5 μg total RNA per lane was denatured for 10 min at 65°C in the loading buffer containing 95% (v/v) formamide and separated on 8% polyacrylamide gels containing 7.6 M urea with a low range ssRNA ladder (New England Biolabs). After separation, RNAs were transferred onto Hybond-N+ membranes (GE Healthcare) by electroblotting and cross-linked to the membrane using UV. Membranes were prehybridized at 42°C, followed by hybridization with 10 pmol 5′-biotin-labeled DNA probes (Supplementary Table S2) for 12 h. After three rounds of washing for 10 min each in 1×, 0.2×, and 0.1× SSC-0.1% SDS solutions, signals were visualized using chemiluminescent nucleic acid detection module (Thermo Scientific) according to the manufacturer’s protocol.

In total, ~41 M unique mapped reads for dRNA-seq libraries and ~63 M for whole-transcript sequencing libraries were collected (Supplementary Table S1).

The cDNA sequences generated from dRNA-seq libraries were enriched at the +1 site of transcription. This enrichment (≥10 reads enriched in the 5′-end (+) libraries) and other plausible criteria described in Methods helped TSS identification. By integrating cDNA mappings from the 5′-end (+) and (−) libraries and the whole-transcript libraries, the sequencing approaches identified the transcription start sites (TSSs) for many of the operons. Examples of TSS identifications are shown in Figure 1A. TSS categories (Figure 1B) were further refined through mapping the whole-transcript cDNA reads to the corresponding 5′-end reads. Manual checking reassigned about 100 gene TSSs (gTSSs) (Supplementary Table S3). Most of the mRNAs in the whole-transcript libraries lacked the 20–30 nt from their proximal 5′ terminus, as reviewed by Wang et al.22. This indicates that the canonical whole-transcript sequencing did not obtain the precise 5′ end (TSS) for most mRNAs.

We detected expression of 2925 and 3068 out of the 3167 original annotated ORFs in the 18°C- and 8°C-whole-transcript cDNA libraries, respectively (Supplementary Table S4). Combining DOOR database22 and Rockhopper methods22 and manual curation as described in Material and Methods, 671 operons (≥2 genes) that comprise 2006 ORFs were predicted (Supplementary Figure S2, Supplementary Table S5). The largest operon was the ribosomal protein complex that comprised of 23 genes with a single gTSS (Supplementary Figure S2A). However, another large operon for archaeal exosome, an RNase complex to mediate 3′→5′ degradation of RNAs23, contained internal TSSs (Supplementary Figure S2B), suggesting a dynamic transcription of suboperons.

By annotation of cDNAs with ≥10 reads enriched in the 5′-end (+) libraries, 2506 and 2735 non-redundant TSSs were identified in the 18°C- and 18°C-transcriptomes, respectively (Figure 1C). Those included 1680 gTSSs for 1534 annotated ORFs (Supplementary Table S3), 195 TSSs for non-coding RNAs (nTSSs) present in 125 intergenic regions (IGRs) (Supplementary Table S6), 1110 aTSSs (Supplementary Table S7) in 908 ORFs and 1440 iTSSs (Supplementary Table S8) inside 1068 ORFs. Promoters including transcription factor IIB recognition element (BRE) and TATA box

Figure 1 | Identification of the primary transcription start sites (TSSs) in M. psychrophilus R15. Using an integrated approach including differential RNA-sequencing (dRNA-seq) and whole-transcript sequencing, TSSs were identified in the temperature-responsive transcriptome of R15 growing at 8 and 18°C. (A) cDNA reads mapped to the metYX operon from dRNA-seq (+) and (−) libraries are shown as red and blue, respectively, and those from the whole-transcript (w) sequencing libraries are shown in brown. (+) indicates a 5′-end library for primary transcripts and (−) indicates a 5′-end library for both primary and processed transcripts. Blue, green, and grey arrows indicate the gene transcription start sites (gTSS), intergenic region TSS (nTSS), and gene-internalexpression transcription TSS (iTSS), respectively. (B) Diagram of TSS categories based on the expression and gene architecture: gene (g), internal (i), antisense (a), and non-coding (n) RNA. Grey lines represent the Illumina reads. (C) Venn diagram of the TSS numbers detected in 18°C outside the parenthesis) and 8°C (inside the parenthesis) libraries. Many TSSs affiliated to multiple categories. A total of 2506 TSSs were identified in the 8°C library, and 2735 TSSs were identified in the 18°C library.

| 5 : 9209 | DOI: 10.1038/srep09209 | www.nature.com/scientificreports
Extreme outliers are depicted by dots, showing that the UTRs distributed at lengths of 300–500 nt are common in R15 Helicobacter pylori transcripts. Here, 15.56 and 16.01% transcripts were leaderless (UTR SCIENTIFIC REPORTS Figure 2|5 transcripts were leaderless (5 Figure 2B) and the longest length of 497 nt. 15.6% and 16% of an ORF. The 5′ UTR lengths of the R15 primary transcripts were mainly 20–150 nt (Figure 2A), with a median length of 55 nt (Figure 2B) and the longest length of 497 nt. 15.6% and 16% of transcripts were leaderless (5′ UTR <10 nt) at 8°C- and 18°C, respectively. About 51% of the 5′ UTRs were >50 nt in length, and 24% were >150 nt. Most of the ribosomal protein mRNAs had longer 5′ UTRs, with an average of 165 nt (Supplementary Table S3).

Interestingly, multiple gTSSs were identified for 219 ORFs (Supplementary Table S9, Figure 3A). Strikingly, 84 ORFs exhibited temperature-dependent gTSS, indicating a temperature-induced gTSS-selection (Supplementary Table S9). For these ORFs, transcripts with different length 5′ UTR, i.e. RNA isoforms were observed. The leaderless mRNAs (with <10 nt 5′ UTR) were more abundant in the 18°C transcriptome, while longer 5′ UTR from 120 to 250 nt were synthesized more frequently at 8°C (Figure 3B). Moreover, for some of these 84 genes, there was a remarkable increase in the lengths of their 5′ UTRs at 8°C (Supplementary Figure S5). For example, Figures 3C and 3E show dRNA-seq detected multi-gTSSs which were verified by 5′-RACE (Figures 3D and 3F).

An Hsp20 gene (Mpsy_0075) exhibited a notable temperature-responsive gTSS-selection, by preferentially using the upstream promoter and TSS at 8°C (Figure 4A and 4B) which was confirmed by primer extension assay (Figure 4C). This resulted in more mRNA isoform with a 65-nt 5′ UTR at 8°C and more with a 12-nt 5′ UTR at 18°C. A secondary structure was predicted in the 65-nt 5′ UTR (Figure 4D), in which ribosomal binding site (RBS) was buried and may hinder translation. Another Hsp20 encoding gene (Mpsy_0869) also exhibited the temperature-related gTSS-selection (Supplementary Figure S6).

Cold-induced sRNAs in the intergenic regions (IGRs). A total of 195 nTSSs were detected in 129 IGRs (Supplementary Table S6). They represented noncoding small RNAs that could exert post-transcriptional regulation of gene expression. 43 IGRs contained ≥2 sRNAs, and some were mutual antisense sRNAs (Figure 5A). sRNAs, including some tRNAs, were among the ten most abundant transcripts in both libraries (Supplementary Table S10), especially the signal recognition particle RNA (SRP RNA, also known as 7S RNA). The abundance of some nTSSs increased dramatically (up to >1000-fold) in the 8°C library (Supplementary Table S6, Figure 5B). The cold-induced expression of some sRNAs was confirmed by Northern blot analysis (Figure 5C). Remarkably, nine highly conserved sRNAs were all induced by cold, and possessed a conserved motif, tentatively named the ‘cold box’, upstream of their promoters (Supplementary Figure S7). These results suggest that sRNAs are involved in the cold adaptation of the archaeon.

dRNA-seq also identified 49 gTSSs for 40 tRNA genes, and many functional sRNAs including a SRP RNA, an RNase P, three C/D box small RNAs, and two group II introns (Supplementary Table S11). Interestingly, dRNA-seq detected the expression of tRNA-Pyl gene (Supplementary Figure S8A), a dedicated pyrrolysine tRNA (tRNA-Pyl) responsible for inserting the necessary pyrrolysine residue during translation of the methylammoniumcorrinoid methyltransferases. The previous automatic annotation failed to identify the tRNA-Pyl in the R15 genome, most probably because of its distant genomic location from the aminoacyl-tRNA synthetase gene (Supplementary Figure S8B). Based on this finding, six methylammoniumcorrinoid methyltransferase encoding genes were re-annotated (Supplementary Figure S8C, Table S12). These genes had been disrupted by the pyrrolysine-encoding amber stop codon in automatic annotation.

Antisense transcriptions. 1110 antisense transcription start sites (aTSS) that complemented 908 protein encoding genes (28.7% of total ORFs) of R15 were detected in the libraries, and promoters were predicted for 622 aTSSs (Supplementary Table S7). Supplementary Figure S9A showed the reads mapping diagrams of aTSS expressions. As shown in Supplementary Table S7, the majority of the aTSSs overlapped internal regions of the sense transcripts, and 245 (22%)...
and 111 (10%) aTSSs overlapped 5’ and 3’ UTRs of the complementary mRNAs, respectively.

For many genes, the temperature-responsive abundances of aTSSs and their targeted mRNAs were either positively or negatively correlated (Supplementary Figure S9B). Expression of aTSS for genes encoding the enzymes key to methylotrophic methanogenesis, *mtaB* (*Mpsy_0909*), *mttC* (*Mpsy_1686*), *cdhE* (*Mpsy_2895*), *hdrBA* (*Mpsy_2939, 2940*), and *mtaC* (*Mpsy_3032*) were upregulated at 8°C, while the transcripts of these genes were reduced at 8°C (Supplementary Table S4). This suggests that antisense-based post-transcriptional regulation may play a role in the response of methanogenesis to cold.

**Gene-internal TSS.** A total of 1068 genes possessed internal TSS, and 753 iTSSs were detected in the 18°C library and 601 in 8°C library. Most of iTSSs were detected at relatively lower abundance than the gTSS (Supplementary Table S8).

About 25% of iTSSs were found located near the 5’ end of the transcript (Supplementary Figure S10A) and some helped identify the precise start codon of a gene and the exact boundary of an ORF.
For instance, iTSS identification refined the automatic annotation-generated atypical 5′-extension in Mpsy_1484 (DNA polymerase PolB) and Mpsy_2175 (F_{420}-ligase) and re-defined the exact boundaries of the ORFs (Supplementary Figure S10B). In this work, a total of 188 ORFs were re-annotated, including four newly found ORFs (Supplementary Table S12). 51 ORFs were corrected through iTSS identification near their 5′-ends.

Approximately 30% of the identified iTSSs were located at the last quarter of the transcripts, and majority of them were also detected as the gTSSs of their downstream ORFs (Supplementary Figure S10B). In this work, a total of 188 ORFs were re-annotated, including four newly found ORFs (Supplementary Table S12). 51 ORFs were corrected through iTSS identification near their 5′-ends.

Interestingly, iTSSs were detected for most signal transduction histidine kinase genes, suggesting an environmental clue-induced gene internal transcription. For example, an iTSS2 at position 26,651 in Mpsy_0031 would produce a truncated protein retaining only the histidine kinase (HisKA) and the receiver (REC) domains but deleting the PAS/PAC sensor-domains (Supplementary Figure S11A). These domains might have a regulatory function in the phosphorylation of the two component system composed by the protein^{35}.

**Discussion**

An atlas of the genome-wide transcription start sites would provide insights into the primary transcriptome for an organism at a given spatio-temporal condition^{13-15}. This study has identified TSSs for...
Blue bullets indicate RNAs that showed genes. 5S rRNA is included as the control. Growing temperatures of their expressions are indicated at the tops of the transcriptions of C/D box sRNAs and cold-induced sRNAs. sRNAs and the than at 18°C.

Bullets indicate the transcription direction of each sRNA. Green bullets represent differentially expressed sRNAs in response to temperature changes. Inside the bullets are the denoted serial numbers of the small representative differentially expressed sRNAs in response to temperature adaptation and the coordination between transcriptional and post-transcriptional regulation.

Precise TSS identification reveals that, similar with another methanoarchaeon M. mazei49, the majority of the mRNAs in M. psychrophilus R15 possess long 5’ UTRs of 20–150 nt. By providing targets of non-coding regulatory RNAs through cis- or trans-actions, large 5’ UTRs provide potential regulatory elements for post-transcriptional regulation. Specifically, 5’ UTRs are predicted to respond to ambient temperatures because of temperature-sensitive base-pair formation56. An example is the E. coli mRNA of cold shock protein CspA, which has a 160 nt-5’ UTR that behaves like a RNA thermometer57. In addition, large 5’ UTRs may act as riboswitches in gene regulation58. Eukaryotic mRNAs generally have long 5’ UTRs, with the average length of ~100 to ~200 nt11, and they are of the biological importance in controlling transcript stability, intracellular localization, differential translation and microRNA mediated gene regulation59. Oncogenes, tumor suppressors and others associated with cell proliferation all tend to express atypically long and complex 5’ UTRs that are involved in subtle regulations of the corresponding genes60.

This study found that 14% of R15 genes used multiple TSSs with individual promoters (Figure 3, Supplementary Table S9). In prokaryotes, multiple TSSs have been reported in only a few bacteria, such as E. coli26 and the cyanobacterium Anabaena12. In contrast, 10–18% of mammalian genes are reported to use multiple TSSs, especially oncogenes, tumor suppressors and genes associated with cell proliferation12. Transcription of a gene from multiple TSSs would produce mRNA isoforms with alternative 5’ UTRs, which contribute to the development of carcinogenesis. Alternative 5’ UTRs for E. coli infA transcript also shows regulatory functions in translation efficiencies and mRNA stability54. Remarkably, 84 genes in R15 possessed cold-responsive selection of gTSSs and promoters and would be expected to transcribe different mRNA isoforms with alternative 5’ UTRs at different temperatures, like the Hsp20 genes. Though a number of transcriptomic studies on prokaryotes have indicated that many genes are differentially expressed in the cold, a conserved motif or cold box has not been found proximal to the prompters2,4,10. Therefore, it is believed that post-transcriptional regulation, including mRNA stability and translation efficiency, make major contributions to cold adaptation of living organisms17. However, the prevalence of alternative 5’ UTRs generated by cold-responsive gTSS selection (transcriptional regulation) identified in this work suggests that coordinated regulation mechanism between transcription and post-transcription may be of importance in the cold adaptation of methanoarchaea.

Non-coding small RNAs (sRNA) have been identified in all the three domains of life and represent important players in post-transcriptional regulation. However, archaeal sRNAs are poorly characterized12. 195 sRNAs were identified in this work (Supplementary Table S6), some are categorized in the most abundant transcripts in R15 (Supplementary Table S10). 40% of the sRNAs were differentially expressed in the cold, and nine conserved sRNAs with a predicted cold box have not been found proximal to the prompters2,4,10. Therefore, it is believed that post-transcriptional regulation, including mRNA stability and translation efficiency, make major contributions to cold adaptation of living organisms17. However, the prevalence of alternative 5’ UTRs generated by cold-responsive gTSS selection (transcriptional regulation) identified in this work suggests that coordinated regulation mechanism between transcription and post-transcription may be of importance in the cold adaptation of methanoarchaea.

Identified functional sRNAs include signal recognition particle (SRP) RNA, which is an essential component of SRP. In Archaea, it delivers proteins to the plasma membrane63. The SRP RNA in R15 is among the most abundant primary transcripts at both temperatures (Supplementary Table S10). This can be related to the 83 proteins that contain signal peptides in M. psychrophilus R15 including S-layer proteins. In addition, C/D box sRNAs involved in 2-O-methylation of rRNA and tRNAs are also abundantly expressed, and their associated proteins, such as fibrillarin (Mpsy_1937), Nop5 (Mpsy_1936) and L7Ae (Mpsy_2863), are all expressed.

![Figure 5](image-url) 

Figure 5 | Non-coding small RNAs in the 5’-end transcriptomes of R15. (A) Schematic of the multiple nTSSs located in one intergenic region (IGR) as the mutual antisense transcripts. (B) Genome locations of the representative differentially expressed sRNAs in response to temperature changes. Inside the bullets are the denoted serial numbers of the small RNAs that are defined as the upstream locus number-sRNA number. Bullets indicate the transcription direction of each sRNA. Green bullets indicate RNA that showed 2-4-fold less expression at 8°C than at 18°C. Blue bullets indicate RNAs that showed 25-35-fold more expression at 8°C than at 18°C. t, tRNA; r, rRNA. (C) Northern blot analysis verified the transcriptions of C/D box sRNAs and cold-induced sRNAs. sRNAs and the growing temperatures of their expressions are indicated at the tops of the gels. 5S rRNA is included as the control.

48.4% of the total annotated ORFs or 91% predicted operons of the psychrophilic methanoarchaeon R15. Multiple TSS per gene and cold-responsive TSS indicate a dynamic transcriptome in this archaeon, thus providing a new avenue for exploring archaean cold adaption and the coordination between transcriptional and post-transcriptional regulation.
Nucleotide modification of RNAs may play a role in temperature adaptation, at least in thermophiles. For instance, ribose methylation levels increase in *S. solfataricus* RNAs at high temperatures. Because there are fewer C/D box sRNAs in *M. psychrophilus*, post-transcriptional modifications of sRNAs may be less important to psychrophiles.

Antisense transcription, which has been found in the majority of prokaryotic and eukaryotic genomes, can affect the overlapping sense transcripts via a double-stranded RNA-dependent mechanism. An RNAi-like function displays mutual exclusion between the sense and paired antisense transcripts. AsRNA-driven mRNA degradation plays a role in post-transcriptional regulation in methanogenic Archaea. Antisense transcription was found in 28.7% of total R15 ORFs, and many were upregulated at 8°C (Supplementary Table S7), indicating their involvements in the cold-adaptation of R15.

In conclusion, this study reveas an unexpectedly dynamic transcriptome in a psychrophilic archaeon in response to cold, shedding light on transcriptional and post-transcriptional regulation in archaeal cold adaption. The findings of this study are also of evolutionary significance. As the *cspA* of *E. coli* employs the long 5' UTR transcript to achieve the post-transcriptional regulation and alternative 5' UTRs are a major regulatory element in mammalian gene expressions, the large and alternative 5' UTRs found in this psychrophilic methanogenic archaeon might hint at an evolutionary conservation between methanogenic Archaea and eukaryotes and demonstrate the importance of post-transcriptional regulation mediated by the 5' UTRs in archaeal gene expression.

1. Cavicchioli, R. Cold-adapted archaea. *Nat. Rev. Microbiol.* 4, 331–343 (2006).
2. Saunders, N. F. et al. Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea *Methanogenium frigidum* and *Methanococoides burtonii*. *Genome Res.* 13, 1580–1588 (2003).
3. Campanaro, S. et al. Temperature-dependent global gene expression in the Antarctic archaeon *Methanococoides burtonii*. *Environ. Microbiol.* 13, 2018–2038 (2011).
4. Nichols, D. S. et al. Cold adaptation in the Antarctic Archaeon *Methanococoides burtonii* involves membrane lipid unsaturation. *J. Bacteriol.* 186, 8508–8515 (2004).
5. Zhang, G., Jiang, N., Liu, X. & Dong, X. Methanogenesis from methanol at low temperatures by a novel psychrophilic methanogen, *Methanolobus psychrophilus* sp. nov., prevalent in Zoige wetland of the Tibetan plateau. *Appl. Environ. Microbiol.* 74, 6114–6120 (2008).
6. Chen, Z., Yu, H., Li, L., Liu, S. & Dong, X. The genome and transcriptome of a new psychrophilic archaeon, *Methanobacterium psychrophilus* R15, reveal its cold adaptive characteristics. *Environ. Microbiol. Rep.* 4, 633–641 (2012).
7. Narberhaus, F. mRNA-mediated detection of environmental conditions. *Arch. Microbiol.* 178, 404–410 (2002).
8. Yamanaka, K., Mita, M. & Inouye, M. Mutation analysis of the 5' untranslated region of the cold shock *cpA* mRNA of *Escherichia coli*. *J. Bacteriol.* 181, 6284–6291 (1999).
9. Gioliodori, A. M. et al. The *cpA* mRNA is a thermosensor that modulates translation of the cold-shock protein *Cpa*. *Mol. Cell.* 37, 21–33 (2010).
10. Cao, Y., Li, J., Jiang, N. & Dong, X. Mechanism for stabilizing mRNAs involved in methanol-dependent methanogenesis of cold-adaptive *Methanocarcina mazeti* zm-15. *Appl. Environ. Microbiol.* 80, 1291–1298 (2014).
11. Mignone, F., Gissi, C., Liuni, S. & Pesole, G. Untranslated regions of mRNAs. *Genome Biol.* 3(3), REVIEW50014 (2002).
12. Smith, L. Post-transcriptional regulation of gene expression by alternative 5' gene expressions, the large and alternative 5' UTRs found in this psychrophilic methanogenic archaeon might hint at an evolutionary conservation between methanogenic Archaea and eukaryotes and demonstrate the importance of post-transcriptional regulation mediated by the 5' UTRs in archaeal gene expression.

Author contributions
J.L., L.Q. and X.D. designed the project; J.L., L.Q., L.Y., Y.P.L., W.Z.G. performed experiments; J.L., L.Q., Y.G., J.W. and X.D. analyzed the data; X.D. wrote the manuscript with input from J.W., J.L., L.Q and W.S. All authors read and approved the final manuscript.

Acknowledgments
This work was supported by National Natural Science foundation of China under no. 31400001 and 31100035.

References
1. Cavicchioli, R. Cold-adapted archaea. *Nat. Rev. Microbiol.* 4, 331–343 (2006).
2. Saunders, N. F. et al. Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea *Methanogenium frigidum* and *Methanococoides burtonii*. *Genome Res.* 13, 1580–1588 (2003).
3. Campanaro, S. et al. Temperature-dependent global gene expression in the Antarctic archaeon *Methanococoides burtonii*. *Environ. Microbiol.* 13, 2018–2038 (2011).
4. Nichols, D. S. et al. Cold adaptation in the Antarctic Archaeon *Methanococoides burtonii* involves membrane lipid unsaturation. *J. Bacteriol.* 186, 8508–8515 (2004).
5. Zhang, G., Jiang, N., Liu, X. & Dong, X. Methanogenesis from methanol at low temperatures by a novel psychrophilic methanogen, *Methanolobus psychrophilus* sp. nov., prevalent in Zoige wetland of the Tibetan plateau. *Appl. Environ. Microbiol.* 74, 6114–6120 (2008).
6. Chen, Z., Yu, H., Li, L., Liu, S. & Dong, X. The genome and transcriptome of a new psychrophilic archaeon, *Methanobacterium psychrophilus* R15, reveal its cold adaptive characteristics. *Environ. Microbiol. Rep.* 4, 633–641 (2012).
7. Narberhaus, F. mRNA-mediated detection of environmental conditions. *Arch. Microbiol.* 178, 404–410 (2002).
8. Yamanaka, K., Mita, M. & Inouye, M. Mutation analysis of the 5' untranslated region of the cold shock *cpA* mRNA of *Escherichia coli*. *J. Bacteriol.* 181, 6284–6291 (1999).
9. Gioliodori, A. M. et al. The *cpA* mRNA is a thermosensor that modulates translation of the cold-shock protein *Cpa*. *Mol. Cell.* 37, 21–33 (2010).
10. Cao, Y., Li, J., Jiang, N. & Dong, X. Mechanism for stabilizing mRNAs involved in methanol-dependent methanogenesis of cold-adaptive *Methanocarcina mazeti* zm-15. *Appl. Environ. Microbiol.* 80, 1291–1298 (2014).
11. Mignone, F., Gissi, C., Liuni, S. & Pesole, G. Untranslated regions of mRNAs. *Genome Biol.* 3(3), REVIEW50014 (2002).
12. Smith, L. Post-transcriptional regulation of gene expression by alternative 5' gene expressions, the large and alternative 5' UTRs found in this psychrophilic methanogenic archaeon might hint at an evolutionary conservation between methanogenic Archaea and eukaryotes and demonstrate the importance of post-transcriptional regulation mediated by the 5' UTRs in archaeal gene expression.

Author contributions
J.L., L.Q. and X.D. designed the project; J.L., L.Q., L.Y., Y.P.L., W.Z.G. performed experiments; J.L., L.Q., Y.G., J.W. and X.D. analyzed the data; X.D. wrote the manuscript with input from J.W., J.L., L.Q and W.S. All authors read and approved the final manuscript.

Acknowledgments
This work was supported by National Natural Science foundation of China under no. 31400001 and 31100035.

References
Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Li, J. et al. Global mapping transcriptional start sites revealed both transcriptional and post-transcriptional regulation of cold adaptation in the methanogenic archaeon Methanolobus psychrophilus. Sci. Rep. 5, 9209; DOI:10.1038/srep09209 (2015).