CORRESPONDENCE

REVISED

Non-human *lnc-DC* orthologs encode Wdnm1-like protein [v2; ref status: indexed, http://f1000r.es/4el]

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Abstract

In a recent publication in *Science*, Wang *et al*. found a long noncoding RNA (lncRNA) expressed in human dendritic cells (DC), which they designated *lnc-DC*. Based on lentivirus-mediated RNA interference (RNAi) experiments in human and murine systems, they concluded that *lnc-DC* is important in differentiation of monocytes into DC. However, Wang *et al*. did not mention that their so-called "mouse *lnc-DC ortholog*" gene was already designated "*Wdnm1-like*" and is known to encode a small secreted protein. We found that incapacitation of the *Wdnm1-like* open reading frame (ORF) is very rare among mammals, with all investigated primates except for hominids having an intact ORF. The null-hypothesis by Wang et al. therefore should have been that the human *lnc-DC* transcript might only represent a non-functional relatively young evolutionary remnant of a protein coding locus. Whether this null-hypothesis can be rejected by the experimental data presented by Wang *et al*. depends in part on the possible off-target (immunogenic or otherwise) effects of their RNAi procedures, which were not exhaustive in regard to the number of analyzed RNAi sequences and control sequences. If, however, the conclusions by Wang *et al*. on their human model are correct, and they may be, current knowledge regarding the *Wdnm1-like* locus suggests an intriguing combination of different functions mediated by transcript and protein in the maturation of several cell types at some point in evolution. We feel that the article by Wang *et al*. tends to be misleading without the discussion presented here.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 30 Sep 2014	*AWAITING PEER REVIEW*	
version 1 published 11 Jul 2014	✓ report	✓ report

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REVISED **Amendments from Version 1**

The excellent referee reports were mostly positive, but included a set of extra details and thoughts. For that we now refer to those reports. We now also mention mass spectrometry reports that support the existence of Wdnm1-like protein.

See referee reports

Correspondence

In their recent publication in *Science*, Wang *et al.*[1] aimed to identify lncRNAs involved in DC differentiation and function. In order to do this they used an established model of human DC differentiation from peripheral blood monocytes (Mo), based on addition of recombinant cytokines. They found that transcription of the human *Wdnm1-like* pseudogene (*Wdnm1-like-ψ*), or *lnc-DC* as they call it, was robustly induced by the Mo-DC differentiation process. Furthermore, they found *Wdnm1-like-ψ* highly transcribed in other dendritic cells, and confirmed correlation of *Wdnm1-like-ψ* transcription with DC differentiation in several ways. To investigate a functional role of *Wdnm1-like-ψ* in their Mo-DC differentiation model, they used a lentivirus-mediated RNA interference (RNAi) system. The RNAi interference with *Wdnm1-like-ψ* fragments resulted in a pronounced effect on Mo-DC differentiation as measured by expression of genes and molecules involved in the immune system, the ability to take up antigen, and the capacity to stimulate T-helper cells. Wang *et al.* showed by a number of experiments that the *Wdnm1-like-ψ* transcript, in particular the 3'-end, has some specificity for binding to the STAT3 transcription factor and can reduce STAT3 dephosphorylation by phosphatase SHP1. And, importantly, they showed that in their human Mo-DC differentiation model the effect of STAT3 inhibition caused similar effects as knockdown of *Wdnm1-like-ψ*. They therefore postulated that human *Wdnm1-like-ψ* transcript is an important regulator of DC differentiation by enhancing STAT3 activity through prevention of STAT3 dephosphorylation by SHP1. The results and human model presented by Wang *et al.* are generally convincing, yet some questions remain, such as to why not for all experiments both "no transfection control" (used in a few experiments) and "control RNAi" (used in all experiments) were included, and why they only used a single RNAi control sequence. RNAi control sequences are relevant because off-target genes might be knocked down (e.g. Jackson *et al.*[3]), but also because the lentivirus system using short hairpin RNA (shRNA) can have immunogenic properties in an shRNA-sequence-dependent manner (e.g. Kenworthy *et al.*[4]). Notably, in some experiments Wang *et al.*[1] independently knocked down two different fragments of *Wdnm1-like-ψ*, with similar experimental results, thus reducing the chance that off-target effects of their RNAi systems influenced their conclusions. On the other hand, since the use of two positive RNAi systems suggests that Wang *et al.* were aware of the potential weaknesses of the system, this raises the question as to why they only used a single sequence for their RNAi control experiments. Regardless, we consider the part of their manuscript on human *Wdnm1-like-ψ* to be mostly solid and interesting, and the main reason why we are so (overly) critical is that acceptance of the model for human *Wdnm1-like-ψ* function as proposed by Wang *et al.* leads to a quite spectacular evolutionary model, as outlined below. Our view, which is supported in the accompanying referee report provided by Dr. Burchard, is that such a spectacular claim requires very robust evidence which in this case probably requires a higher number of RNAi controls.

Whereas the presentation of their human data appears to be mostly correct, we feel that the way Wang *et al.*[1] present their mouse model is inappropriate. Wang *et al.* used a mouse model to confirm that knockdown of *Wdnm1-like(-ψ)* results in impaired DC differentiation. Technically these experiments in mice worked as they expected, indicated both by *in vitro* and *in vivo* results, and they also found that knockdown of murine *Wdnm1-like* could lead to reduction of STAT3 phosphorylation, although they apparently did not check if murine *Wdnm1-like* transcript can bind STAT3. However, even though Wang *et al.* refer to Gene symbol 110000G20Rik which mentions "Wdnm1-like", they only present the readers with the term "mouse lnc-DC ortholog". This is highly misleading as it suggests that the transcript also relates to a long noncoding RNA in mice. The authors even state "*Taken together, our data suggest that lnc-DC is vital for DC differentiation in both human and mice*". However, in mice the gene encodes a functional Wdnm1-like protein, as shown by recombinant analysis[2], and our extensive analysis of mammalian sequence databases indicates that the *Wdnm1-like* ORF incapacitation is very rare among mammals. Actually, among the eutherian mammals that we investigated and for which the relevant genomic region information was available, only humans (and Neanderthals and Denisovans) lacked the capacity to encode the otherwise highly conserved Wdnm1-like protein sequence (Figure 1). At the level of the genus *Pan* (chimpanzee and bonobo) the N-terminus of the predicted mature protein differs from consensus, but even in gorilla and orangutan the encoded Wdnm1-like protein appears fully normal. So possibly the function of the Wdnm1-like protein started to lose importance after separation of *Homo*/*Pan* from the other apes, which is quite recent in evolutionary terms. Calculation of synonymous (ds) versus nonsynonymous (dn) nucleotide substitution rates, using software available at http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html, indicates conservation of Wdnm1-like protein function after most of the animals shown in Figure 1 had separated in evolution. Namely, in pairwise comparisons, for the depicted set of eutherian mammals except *Pan*/*Homo* the average ds/dn ratio is 3.5, and for the set of primates except *Pan*/*Homo* this value is 3.0. Thus, although in each individual species experimental evidence would still be required, it is expected that most eutherian mammals possess functional Wdnm1-like protein. Probably because of lack of directed investigations, naturally expressed endogenous full-size Wdnm1-like proteins have not yet been reported. However, our search of the PeptideAtlas database of peptides identified by mass spectrometry identified a rat (*Rattus Norwegicus*) Wdnm1-like fragment encoded by properly spliced *Wdnm1-like* transcript (http://www.peptideatlas.org, peptide PAp03984316).

The name *Wdnm1-like* was first coined by Adachi *et al.*[5], who found that *Wdnm1-like* transcript was differentially expressed in limbal versus central corneal epithelia in rat, and who observed similarity of the encoded protein with Wdnm1. Within the serial analysis of gene expression (SAGE) experiment by Adachi *et al.*[5], *Wdnm1-like* comprised the most abundant SAGE tag present exclusively in the limbal library, and the authors hypothesized that Wdnm1-like might be a marker of limbal stem cells. They could, however, not rule out

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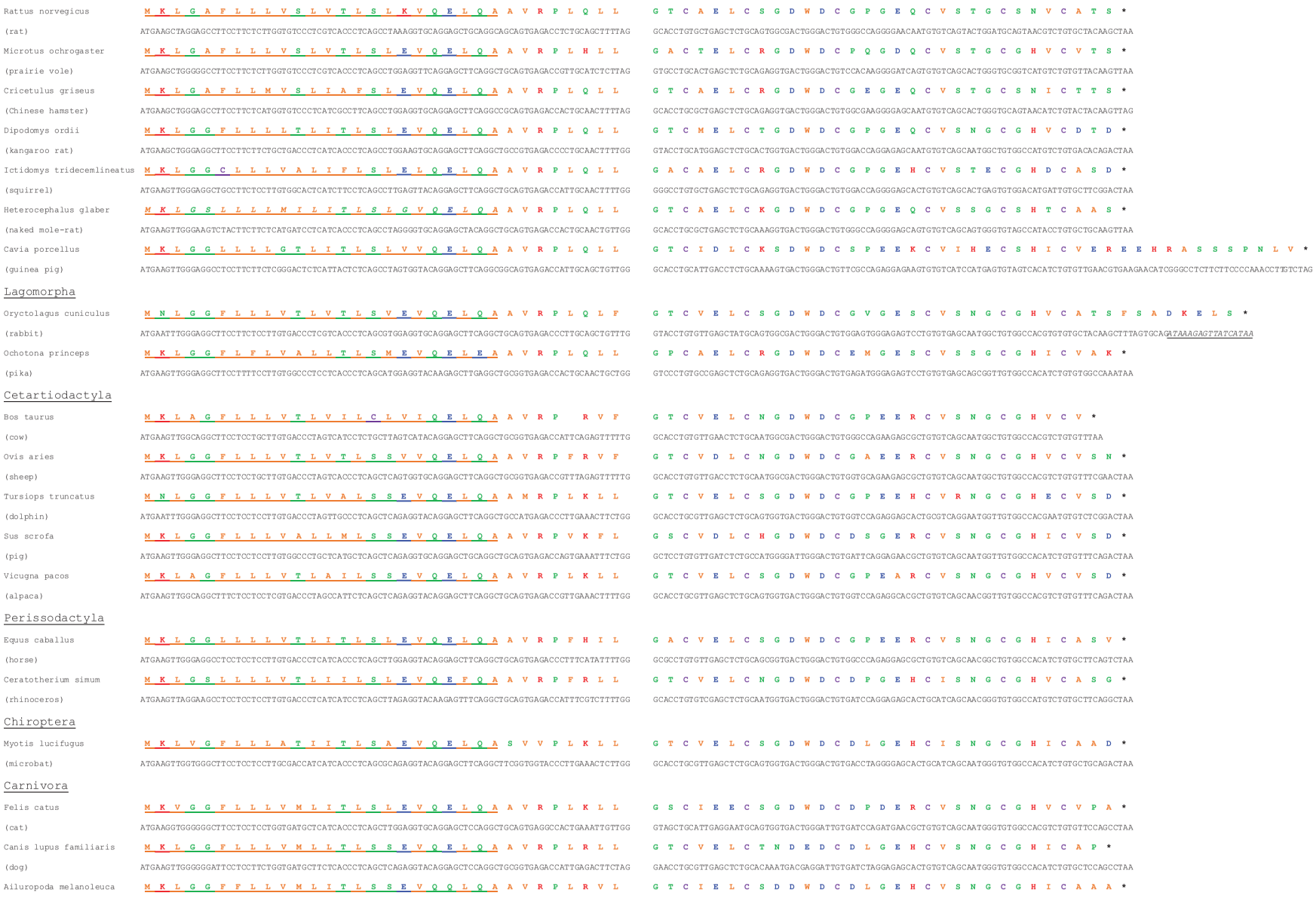

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Figure 1. The highly conserved coding sequences of mammalian *Wdnm1-like*. The figure shows deduced Wdnm1-like amino acid sequences plus their coding nucleotide sequences in representative mammals.

After evolutionary separation from gorilla, in an ancestor common to the genera *Pan* (including chimpanzee and bonobo) and *Homo* (including human, Neanderthal and Denisovan), the nucleotide region coding the N-terminus of the mature Wdnm1-like protein was modified by deletions (yellow shading). Nevertheless, in the genus *Pan* the *Wdnm1-like* open reading frame (ORF) remained intact. Only in *Homo* the Wdnm1-like coding sequence was interrupted by a frameshift through a single nucleotide deletion (red shading) within the leader peptide coding region (the resulting change in amino acids is shaded grey). For the human *Wdnm1-like* locus several transcripts (splicoforms) were found (Ensembl reports ENST00000590346, ENST00000588180, ENST00000587298, ENST00000590012, ENST00000589987, ENST00000592556, ENST00000566140, and ENST00000589777); however, we agree with Wang *et al.*[1] that software investigation of the known transcripts suggests that the human *Wdnm1-like* locus does not code a functional protein (analyses not shown).

The marsupial *Monodelphis domestica* (opossum) was the only non-eutherian mammal for which we could identify *Wdnm1-like*, situated upstream of the gene *HEAT Repeat Containing 6* (*HEATR6*) like its ortholog in eutherian mammals. To avoid gaps in the bulk of the figure, the N-terminus of the opossum sequence is not perfectly aligned with Wdnm1-like of eutherian mammals.

Except for rabbit (see Methods section), the figure shows the ORFs of sequences corresponding to the murine Wdnm1-like protein coding transcript of NCBI accession NM_183249, while other (possible) splicoforms are neglected. The intron site is indicated by a downward triangle. Intron sequences are not shown, but the below listed genomic sequence reports agree with GT-AG borders. For most of the species, the depicted sequences were supported by transcript reports, as exemplified per species in the Methods section. In the figure, dashes indicate gaps that were introduced for optimal sequence alignment. The alignments were performed by hand.

Amino acid sequences are indicated above the second nucleotides of codons. Basic residues are indicated in red, acidic residues in blue, and green residues are more hydrophilic than the orange ones (following reference[9]). Cysteines are in violet. Asterisks correspond with stop codons. Predicted leader sequences are underlined.

The mouse *Wdnm1-like* sequence was designated “mouse lnc-DC ortholog” by Wang *et al.*[1], and they targeted the regions shaded blue and green for transcript knockdown by “RNAi-1” and “RNAi-2”, respectively, using a lentivirus-mediated RNA interference system.

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the possibility that Wdnm1-like was expressed by other cell types present in limbal epithelia, such as for example dendritic cells. A later study on rodent *Wdnm1-like* was performed in mice by Wu and Smas[2]. Wu and Smas got interested in *Wdnm1-like* after they found it highly upregulated upon differentiation of preadipocytes into adipocytes. They found *Wdnm1-like* to be selectively expressed in liver and adipose tissue, and enriched in white adipose depots versus brown. Recombinant expression of tagged murine Wdnm1-like in HT1080 human fibrosarcoma cells revealed a small secreted protein[2]. Because Wdnm1-like is a distant member of the whey acidic protein/four-disulfide core (WAP/4-DSC) family, of which several members have roles as proteinase inhibitors, Wu and Smas speculated that Wdnm1-like might have a similar function. An important class of extracellular proteases involved in adipocyte differentiation are the matrix metalloproteinases (MMPs), which can degrade extracellular matrix (ECM) components. Therefore, Wu and Smas investigated whether MMPs expressed by HT1080 were affected by the recombinant Wdnm1-like expression, and they found an increased amount of the active form of MMP-2[2]. Thus, rather than having an inhibitory effect, Wdnm1-like appears to enhance activation of a protease. Wu and Smas conclude with "*Future studies are required to address the mechanism(s) underlying the function and regulation of adipocyte-secreted Wdnm1-like*"[2], and according to literature this situation has not changed since then.

Looking at the combined publications, a very complicated picture emerges. In most mammals the *Wdnm1-like* locus encodes a protein, with humans as an exception which is possibly unique. In rat, *Wdnm1-like* is differentially expressed in limbal versus central corneal epithelia[4]. In mouse, *Wdnm1-like* is expressed upon adipogenesis, and Wdnm1-like protein enhances the production of active MMP-2[2]. In human and mouse, the *Wdnm1-like(-ψ)* transcript appears functionally associated with dendritic cell differentiation, and at least in humans this may be mediated by binding of the transcript to STAT3[1]. This leaves questions for future research such as, for example, whether human *Wdnm1-like-ψ* transcript is also associated with adipogenesis, and whether murine *Wdnm1-like* transcript exerts its function on DC differentiation by binding to STAT3 or by encoding Wdnm1-like protein. Supporting that the Wdnm1-like proteins and transcripts in some extinct or extant animals may have (had) synergetic functions, is the fact that differentiation of both adipocytes and limbal epithelial cells can involve STAT3[6,7]. So, despite our points of criticism, we think that the results and human model by Wang *et al.* may be valid and part of a more complex evolutionary scenario that involves distinct functions at the transcript and protein level, and a number of different tissues and cell types. In general, we think that studies on long noncoding RNAs typically require discussion of the evolutionary context[8], especially when dealing with wide species borders such as between human and mouse.

Additional note 1

A nice speculation allowed by the combined referenced articles is that Wdnm1-like protein might promote Mo-DC differentiation in humans. After all, murine Wdnm1-like protein was found to enhance MMP-2 activity of human HT1080 cells[2], concluding that humans did not lose their sensitivity to Wdnm1-like protein.

Additional note 2

We did not feel comfortable with the amount of space and visibility the editors of the journal *Science* were able to offer us via their commenting mechanism for the discussion presented here. Therefore, we declined their offer, and instead deemed publication in *F1000Research* a more appropriate vehicle. Through F1000Research we asked specialists and the corresponding authors of several of the referenced articles, including the article by Wang *et al.*, to provide referee reports (which may also include broad views) on our discussion. We are pleased with the excellent referee reports received from Dr. Smas and Dr. Ren, and Dr. Burchard, and recommend them to the readers of our article. Importantly, Drs. Smas and Ren confirmed that we correctly summarized reports on rodent *Wdnm1-like* and listed additional evidence to that matter, while Dr. Burchard substantiated and detailed our notion that the RNAi experiments by Wang *et al.* were inconsistent and probably incomplete. We would welcome comments from the group of Wang *et al.* and also encourage other researchers to leave comments.

Additional note 3

Wdnm1-like protein appears to be very interesting. Not only may it be involved in differentiation of several cell types, it also is intriguing because it appears highly conserved throughout eutherian mammals and (rather) uniquely lost in hominids. It may help determine what makes us human.

Methods

The partial *Wdnm1-like* sequence information available for extinct hominids, namely, for Neanderthal and Denisovan, was retrieved using the UCSC genome browser (http://genome.ucsc.edu). All other sequences shown in the figure were retrieved from Ensembl (www.ensembl.org/) or NCBI (http://www.ncbi.nlm.nih.gov/) databases. For a representative list of model species, we investigated database sequences of all mammals for which genomic sequences are available in the Ensembl database, and also of *Pan paniscus* (bonobo). For some of those animals sequence information for the *Wdnm1-like* ORF or for its expected genomic site was incomplete, and in such case the sequence is not included in the alignment figure.

Leader peptide sequences were predicted by SignalP software (www.cbs.dtu.dk/services/SignalP/) and are underlined. For the species *Panio anubis* (olive baboon), *Heterocephalus glaber* (naked mole-rat), *Myotis lucifugus* (microbat), and *Dasypus novemcintus* (armadillo), besides the here indicated evolutionary conserved cleavage site, the SignalP software also predicted an alternative cleavage site with a calculated higher likelihood (not shown).

Species-specific information related to sequences depicted in Figure 1:

Homo sapiens (human)

The depicted human *Wdnm1-like* pseudogene sequence maps within Ensembl database GRCh37, Chr.17 positions 58162470-to-58165647, reverse orientation. Furthermore, the depicted sequence corresponds with positions 182-to-366 of the transcript sequence of NCBI accession NR_030732.1.

Homo sapiens (Neanderthal) (whether Neanderthal should be considered a subspecies of *Homo sapiens* is a matter of debate)

The depicted Neanderthal sequence was identified from genomic DNA fragments (Read names: M_SL-XAT_0004_FC30PM-DAAXX:1:87:384:343, M_BIOLAB29_Run_PE51_1:2:9:981:262 and C_M_SOLEXA-GA04_JK_PE_SL21:8:99:944:526) isolated from the Vi33.16 and Vi33.25 Neanderthal samples[10] using the UCSC genome browser by comparison with the human Wdnm1-like sequence. The depicted sequence fragment is identical to that of human.

Homo sapiens (Denisovan) (whether Denisovan should be considered a subspecies of *Homo sapiens* is a matter of debate)

The depicted Denisovan sequence was obtained as described for the Neanderthal sequences, and corresponds to part of the read M_SOLEXA-GA02_00040_PEdi_MM_3:8:112:19220:10730#AACCATG,CTCGATG (Meyer *et al.*[11]). The depicted sequence fragment is identical to that of human.

Pan troglodytes (chimpanzee)

The depicted chimpanzee *Wdnm1-like* sequence maps within Ensembl database CHIMP2.1.4, Chr.17 positions 588018940-to-58805072, reverse orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|DRR003370.54864751.1 of experiment set DRX002694.

Pan paniscus (bonobo, or pygmy chimpanzee)

The depicted bonobo *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AJFE01016111.1|, positions 11414-to-14585, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR873628.59588401.2 of experiment set SRX290737.

Gorilla gorilla (gorilla)

The depicted gorilla *Wdnm1-like* sequence maps within Ensembl database gorGor3.1, Chr.5 positions 23775798-to-23778981, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR306801.5146816.1 of experiment set SRX081945.

Pongo abelii (Sumatran orangutan)

The depicted orangutan *Wdnm1-like* sequence maps within Ensembl database PPYG2, Chr.17 32711864-to-32715478, forward orientation. This is a recent intrachromosomal duplication of the original *Wdnm1-like* gene. The Ensembl database shows that Sumatran orangutan still has at least part of that original *Wdnm1-like* gene upstream of *HEATR6*, but information of that region is incomplete. Evidence for transcription of *Wdnm1-like* in orangutan is provided by NCBI SRA database sequence reports *for Pongo pygmaeus* (Bornean orangutan), such as for example gnl|SRA|SRR306799.12707499.1 of experiment set SRX081943.

Nomascus leucogenys (gibbon)

The depicted gibbon *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|ADFV01146912.1|, positions 1414-to-4561, reverse orientation.

Papio anubis (olive baboon)

The depicted olive *Wdnm1-like* sequence maps within Ensembl database Panu_2.0, scaffold JH685681 positions 60156-to-64601, reverse orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR1045089.118535973.1 of experiment set SRR1045089.

Macaca mulatta (rhesus macaque)

The depicted rhesus macaque *Wdnm1-like* sequence maps within Ensembl database MMUL_1, scaffold 1099548049739 positions 121534-to-124737, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR1240160.28991243.2 of experiment set SRR1240160.

Macaca fascicularis (crab-eating macaque)

The depicted crab-eating macaque *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AEHL01027073.1|, positions 5255-to-8524, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|DRR001354.3296367.1 of experiment set DRX000951.

Chlorocebus sabaeus (vervet monkey)

The depicted *Wdnm1-like* sequence maps within Ensembl database ChlSab1.0, Chr.16 positions 29807079-to-29810262, reverse orientation. Transcription is supported by NCBI SRA database sequence reports for the closely related species *Chlorocebus aethiops* (green monkey), such as for example gnl|SRA|SRR1178509.592424.2 of experiment set SRR1178509.

Saimiri boliviensis (Bolivian squirrel monkey)

The depicted squirrel monkey *Wdnm1-like* sequence maps within Ensembl database SalBol1.0, scaffold JH378137 positions 636410-to-639575, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR500949.3269772.2 of experiment set SRX149650.

Callithrix jacchus (marmoset)

The depicted marmoset *Wdnm1-like* sequence maps within Ensembl database C_jacchus3.2.1, Chr.5 positions 88345809-to-88348914, reverse orientation. Transcription is supported by NCBI database accession gb|GAMR01043615.1|.

Tarsius syrichta (tarsier)

The depicted tarsier *Wdnm1-like* sequence maps within Ensembl database tarSyr1, scaffold_1716 positions 51738-to-55873, reverse orientation.

Otolemur_garnettii (bushbaby)

The depicted bushbaby *Wdnm1-like* sequence maps within Ensembl database OtoGar3, scaffold GL873613 positions 7509108-to-7514627, reverse orientation.

Microcebus murinus (mouse lemur)

The depicted mouse lemur *Wdnm1-like* sequence maps within Ensembl database micMur1, GeneScaffold_1067 positions 49762-to-53887, reverse orientation. Transcription is supported by NCBI SRA database

sequence reports, such as for example gnl|SRA|SRR832933.720157201.1 of experiment set SRX270644.

Tupaia chinensis (Chinese tree shrew)

The depicted Chinese tree shrew *Wdnm1-like* sequence maps within Ensembl database TREESHREW, scaffold_15853 positions 2941-to-6216, reverse orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR518934.53798716.1 of experiment set SRX157966.

Mus musculus (mouse)

The depicted mouse *Wdnm1-like* sequence maps within Ensembl database GRCm38, Chr.11 positions 83747027-to-83749327, forward orientation. Transcription is supported by for example NCBI accession NM_183249.1.

Rattus norvegicus (rat)

The depicted rat *Wdnm1-like* sequence maps within Ensembl database Rnor_5.0, Chr.10 positions 70671110-to-70673427, forward orientation. Transcription is supported by for example NCBI accession gb|EF122001.1|.

Microtus ochrogaster (prairie vole)

The depicted prairie vole *Wdnm1-like* sequence maps within Ensembl database MicOch1.0, Chr.7 positions 15310620-to-15312860, reverse orientation. According to the Ensembl database the prairie vole also has an intronless copy of *Wdnm1-like* gene on Chr.X (not shown). Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR058428.108679.2 of experiment set SRX018513.

Cricetulus griseus (Chinese hamster)

The depicted hamster *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AMDS01007412.1|, positions 15363-to-17750, forward orientation.

Dipodomys ordii (kangaroo rat)

The depicted kangaroo rat *Wdnm1-like* sequence maps within Ensembl database dipOrd1, scaffold_2778 positions 48464-to-52516, reverse orientation.

Ictidomys tridecemlineatus (thirteen-lined ground squirrel)

The depicted squirrel *Wdnm1-like* sequence maps within Ensembl database spetri2, scaffold JH393300 positions 533158-to-536139, forward orientation.

Heterocephalus glaber (naked mole-rat)

The depicted naked mole-rat *Wdnm1-like* sequence maps within Ensembl database HetGla_female_1.0, scaffold JH602188 positions 3555009-to-3557720, forward orientation.

Cavia porcellus (domestic guinea pig)

The depicted guinea pig *Wdnm1-like* sequence maps within Ensembl database cavPor3, scaffold_32 positions 10788821-to-10791159, reverse orientation.

Oryctolagus cuniculus (rabbit)

The depicted rabbit *Wdnm1-like* sequence maps within Ensembl database OryCun2.0, Chr.19 positions 25079843-to-25084775, forward orientation. The underlined part in Italic font at the 3'end belongs to a third exon. Transcription is supported by for example NCBI accession gb|GBCH01008538.1|.

Ochotona princeps (pika)

The depicted pika *Wdnm1-like* sequence maps within Ensembl database OchPri3, scaffold JH802106 positions 113719-to-116807, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR850200.108627.2 of experiment set SRX277346.

Bos taurus (cattle)

The depicted cattle *Wdnm1-like* sequence maps within Ensembl database UMD3.1, Chr.19 positions 14485956-to-14490393, reverse orientation. Transcription is supported by for example NCBI accession gb|AW484602.1|.

Ovis aries (sheep)

The depicted sheep *Wdnm1-like* sequence maps within Ensembl database Oar_v3.1, Chr.11 positions 13759463-to-13763966, reverse orientation. Transcription is supported by for example NCBI accession gb|CK830678.1|.

Tursiops truncatus (dolphin)

The depicted dolphin *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|ABRN02348024.1|, positions 2742-to-5945, forward orientation.

Sus scrofa (pig)

The depicted pig *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AJKK01193461.1|, positions 3786-to-7100, reverse orientation. Transcription is supported by for example NCBI accession dbj|AK399701.1|.

Vicugna pacos (alpaca)

The depicted alpaca *Wdnm1-like* sequence maps within Ensembl database vicPac1, GeneScaffold_1352 positions 716864-to-719675, reverse orientation.

Equus caballus (horse)

The depicted horse Wdnm1-like sequence maps within Ensembl database EquCab2, Chr.11 positions 36986601-to-36989473, reverse orientation. The Ensembl database indicates additional copies of *Wdnm1-like* on Chr.11 (not shown). Transcription is supported by for example NCBI accession gb|DN508620.1|.

Ceratotherium simum (rhinoceros)

The depicted rhinoceros *Wdnm1-like* sequence maps within Ensembl database CerSimSim1, scaffold JH767772 positions 17445128-to-17447968, reverse orientation.

Myotis lucifugus (microbat)

The depicted microbat *Wdnm1-like* sequence maps within Ensembl database Myoluc2.0, scaffold_GL430154 positions 92198-to-94525, reverse orientation.

Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR1013468.27145136.1 of experiment set SRR1013468.

Felis catus (cat)
The depicted cat *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AANG02057756.1|, positions 9507-to-13123, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR835496.27404932.1 of experiment set SRX272142.

Canis lupus familiaris (dog)
The depicted dog *Wdnm1-like* sequence maps within Ensembl database CanFam3.1, Chr.9 positions 37619501-to-37622242, reverse orientation. Transcription is supported by for example NCBI accession gb|DR107055.1|.

Ailuropoda melanoleuca (panda)
The depicted panda *Wdnm1-like* sequence maps within Ensembl database ailMel1, scaffold GL193203 positions 54404-to-57462, forward orientation.

Mustela putorius (ferret)
The depicted ferret *Wdnm1-like* sequence maps within Ensembl database MusPutFur1.0, scaffold GL896917 positions 9435086-to-9438171, forward orientation. Transcription is supported by for example NCBI accession gb|JR792458.1|.

Echinops telfairi (lesser hedgehog tenrec)
The depicted tenrec *Wdnm1-like* sequence maps within the genomic sequence of NCBI database accession gb|AAIY02150441.1|, positions 1061-to-5393, forward orientation.

Loxodonta africana (elephant)
The depicted elephant *Wdnm1-like* sequence maps within Ensembl database loxAfr3, scaffold_31 positions 5685863-to-5689773, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR1041765.37646273.1 of experiment set SRR1041765.

Orycteropus afer (aardvark)
The depicted aardvark *Wdnm1-like* sequence maps within Ensembl database OryAfe1, scaffold JH863914 positions 5948889-to-5951515, reverse orientation.

Dasypus novemcinctus (nine-banded armadillo)
The depicted armadillo *Wdnm1-like* sequence maps within Ensembl database Dasnov3.0, scaffold JH562945 positions 1888971-to-1892017, forward orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR494776.6845635.2 of experiment set SRX146634.

Monodelphis domestica (opossum)
The depicted opossum *Wdnm1-like* sequence maps within Ensembl database BROADO5, Chr.2 positions 498828348-to-498830609, reverse orientation. Transcription is supported by NCBI SRA database sequence reports, such as for example gnl|SRA|SRR908062.57922637.2, gnl|SRA|SRR873400.62402918.1, gnl|SRA|SRR943348.21681365, gnl|SRA|SRR943348.11424624, and gnl|SRA|SRR943348.9801988 of experiment sets SRX310006 and SRX290643 (because other *Wdnm1-like* transcript information appears lacking for marsupials, we here provide SRA database accessions that together cover the full ORF).

Author contributions
JMD did most of the research and wrote the manuscript. KTB analyzed sequence databases of extinct hominids and carefully checked the manuscript.

Competing interests
No competing interests were disclosed.

Grant information
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Julja Burchard
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This item of correspondence is in reference to Wang *et al.* (2014), concerning possible cytosolic protein-binding function in conventional dendritic cells by a human noncoding RNA and its mouse ortholog. The correspondents note that the lncRNA designated “lnc-DC” by Wang *et al.* is a protein-coding transcript, Wfdc21 or Wdnm1-like, in all mammals with available sequence other than hominids (*Homo sapiens sapiens*, *Neanderthal* and *Denisova*), and suggest that Wang *et al.* would have been well advised to discuss this information, as they propose functions for the transcript in human and mouse cells.

The correspondent authors provide an abbreviated summary of the original article in the abstract with suitable detail in the body of the letter. The letter brings up an intriguing point worthy of discussion. Drs. Dijkstra and Ballingall frame and present their observations well and do a thorough job assembling and aligning sequences in support. To their point, recent work finds that only 0.3% of anthropoid-specific constrained sequences – functional primate innovations – are coding (del Rosario *et al*., 2014). If a similar proportion holds for significant functions hominids have ceased to perform, Wang *et al.* have identified an unusual case and have not highlighted it as such.

A few items of follow-up may be of interest to clarify and extend the evolutionary observations in this correspondence.

1. Is the frameshift in the erstwhile signal peptide coding sequence in the human reference genome reproduced in all 1000-genomes data, or is WFDC21P a polymorphic human pseudogene?

2. Is guidance by the human reference sequence in assembly of *Neanderthal* and *Denisova* genomes a potential factor in their reproduction of the reported human frameshift?

3. Is there human genetic variation at this locus tied to variation in trait expression, or alternatively is there evidence that variation in this small gene is sufficiently suppressed to leave no functional variation for genetic association studies to mine? Either could be consistent with a significant role in immune function as proposed by Wang *et al.*

4. What is found at the position syntenic to Wfdc21 in marsupials other than the one noted as sharing this gene, and in lower model organisms? This may help clarify the nature of the apparent mammalian innovation at this locus.

5. Do regulatory elements for WFDC21P differ between hominids and other species? If Wfdc21 is a gene with active RNA and protein products, their function will have evolved in the context of cell-type specific expression. Fantom5 CAGE tag data suggests a difference in regulation of human WFDC21P vs. mouse Wfdc21. Although parallel samples are not available for all tissues and cell types, mouse data show strongest expression in myeloid suppressor cells with significant expression also in liver and skin, while human data show 1000x lower maximum expression with best expression in migratory Langerhans cells.

6. Do the sections of WFDC21P-RNA highlighted as functional by Wang *et al.* show signs of hominid or mammalian constraint? It is intriguing that Wang *et al.* show data suggesting STAT3 binding by the 3'-end of WFC21P-RNA in a section downstream of the ORF and thus potentially available for secondary function.

The correspondent authors also discuss the technical merits of the work presented in Wang *et al.,* as indicated by the potential novelty of the findings. Four additional points may be made here.

1. It is important to establish the RNA-dependence of activities discussed by Wang *et al*. While the correspondents are satisfied that lack of human protein coding has been demonstrated, some experiments could be clearer. Wfdc21 is a secreted protein. Did Wang *et al.* examine the supernatant as well as the cells in which Flag-tagged fusions were expressed?

2. The correspondents comment on the inconsistent use of controls. Indeed, the sole controlled experiment suggesting RNA-protein association appears to be the pulldown of STAT3 with biotinylated WFDC21P RNA, with specific absence of the STAT3 band in the antisense control. While RIP with STAT3 experimental and STAT1 control antibodies was conducted, no sequencing is reported so the specificity of interaction with STAT3 is not known. Further, figures on RNA-FISH visualization of association of STAT3p with WFDC21P-RNA do not show controls.

3. Wang *et al.* rely on inhibitors to demonstrate WFDC21P-RNA function. As the correspondents note, one shRNA sequence is primarily employed and it is not consistently paired with varied on- or off-target controls. Literature on functional siRNA screens suggests that a half dozen RNAi sequences with independent seeds are required for dissociation of off- and on-target activities. Further, Wang *et al.* have performed expression profiling on shRNA-treated cells. The profiles can be examined for seed-based off-target activity and for inflammatory response to the lentiviral vector according to published methods. It will be important to establish whether the dendritic cell proteins whose differential expression is highlighted by Wang *et al.* show shRNA-matching seed sequences in their 3'UTRs or respond to lentiviral infection.

4. Wang *et al.* also use published STAT3 inhibitors to elucidate the role of WFDC21P-RNA. It would be intriguing to speculate that an RNA-protein interaction site helps to define the STAT3 binding site of published inhibitor S3I-201 and its effects on STAT3 activity. However, the supplementary material provided by Wang *et al.* show much more profound effects on cytokine production by small molecules than shown for WFDC21P shRNA in the main paper, although effects on T-cell activation remain similar.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 10 Sep 2014

Johannes M. Dijkstra, Fujita Health University, Japan

Dear Dr. Burchard,

We thank you for your extensive and valuable comments. Like the comments by Drs. Smas and Ren, we embrace them as generally positive. Your comments add accuracy to our story, especially regarding the technical part of the RNA investigation by Wang and co-workers.

You wonder, as do Drs. Smas and Ren, and as do we, whether in some individuals or under some conditions, humans may express Wdnm1-like protein. After all, the sequence for the mature protein appears intact, and requirements for a leader peptide are not very unique. However, our rather extensive database investigations could not retrieve a human sequence expected to encode a functional Wdnm1-like protein. Identical frameshifts in Neanderthal and Denisovan *Wdnm1-like* sequence reports, for the reliability of which we have to depend on the respective authors, argue against the likeliness of functional/nonfunctional Wdnm1-like polymorphism in modern humans. An expressed sequence tag (EST), reported as GenBank accession CD692402, suggests that an individual from southern China may have a protein coding *Wdnm1-like* sequence; however, besides repair of the frame-shift in the leader coding region, this sequence has an additional unique modification, and the sequence report may contain technical errors. In short, we could not obtain evidence for intact Wdnm1-like coding sequences in humans, but cannot exclude the possibility that such sequences exist. We would welcome if anyone could provide such evidence or indications.

We took a brief look at Wdnm1-like evolution beyond eutherian mammals. However, except for the mentioned case in opossum, at this evolutionary distance it becomes difficult to distinguish Wdnm1-like orthologues from other family members, and it would become more a discussion on the evolution of Wdnm1-like plus related molecules than of Wdnm1-like alone. Because the primary goal of our study is the discussion of the Wang *et al.* article, which is confined to eutherian mammals, we feel a discussion of deeper Wdnm1-like evolution would make our study too complicated.

Although we did not make systematic comparisons among various genes, we feel that overall the 3'-end region of human *Wdnm1-like-y* transcript which is believed to interact with STAT3, is not especially well conserved among mammals. However, without knowing the precise sequence motif or RNA secondary structure important for that binding, we probably shouldn't speculate on presence or absence of evolutionary constraints on that region.

You raise four additional points regarding the technical merits of the work presented by Wang and co-workers. Importantly, you agree with us that the Wang *et al.* study was inconsistent and probably incomplete in the use of controls. Some points you raise are valid speculations and questions, whereas others can be considered as criticism of the Wang *et al.* article. In our opinion that criticism is mostly right. However, we prefer not to change the open style of our technical comments, and hope that the readers will find the specific issues that you raise when reading your report.

Thank you again for your hard and valuable work.

Sincerely,

Johannes M. Dijkstra and Keith T. Ballingall

Competing Interests: No competing interests were disclosed.

Referee Report 21 August 2014

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Cynthia Smas, **Gang Ren**
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This laboratory (Smas) published a manuscript on the *Wdnm1-like* murine gene in 2008[1]; as such we have been invited to review and comment here on the paper by Dijkstra and Ballingall. Their report presents extensive sequence analysis, for a range of species, of the *Wdnm1-like* gene. The title of the report is an accurate representation of the content of the article and the important points are well-summarized in the abstract. The extensive sequence information they provide in their text is in a format that allows the reader to reassess the sequence analysis data if so desired.

The information provided by Dijkstra and Ballingall makes it abundantly clear that the *Wdnm1-like* locus is predicted to contain a *bona fide* open reading frame that would encode a small secreted protein in all extant species examined except humans. This protein is Wdnm1-like, a member of the WFDC (Whey Acidic Protein Four Disulfide Core) protein family. In all but hominids, the predicted protein encoded by the *Wdnm1-like* gene, also recently annotated in the Gene NCBI database as *Wfdc21*, has clear homology with the WFDC protein family[2]. In hominids (*Homo sapiens*) this locus contains the Wdnm1-like pseudogene, which Wang and co-authors report encodes a long non-coding RNA that they have named *lnc-DC*. In humans, a one base pair deletion is present near the start of what would have been the open reading frame for the Wdnm1-like protein. This frame shift apparently eliminates the protein coding ability of the human gene. However, restoration of the correct open reading frame would be predicted to generate an encoded protein with significant homology to the murine Wdnm1-like protein.

The detailed sequence analyses and other information provided by Dijkstra and Ballingall is valuable to the long-noncoding RNA research community and to those following the recent research into dendritic cell differentiation, in respect to the *Science* report that was published in April 2014 by Wang and co-authors "*The STAT3-Binding Long Noncoding RNA lnc-DC Controls Human Dendritic Cell Differentiation*" [3]. In regard to the *Science* report by Wang and co-authors the design, methods and analysis of the results in the paper by Dijkstra and Ballingall are well-explained and are they appropriate to the topic. The conclusions they reach are sensible, generally balanced and justified. In a few instances, however, the tone of the writing is a bit aggressive. As further discussed below, it appears an assumption is made by Dijkstra and Ballingall that Wang and co-authors were aware of the distinction that the human locus encodes a pseudogene, while in mouse this locus encodes the Wdnm1-like protein.

This laboratory (Smas) identified murine *Wdnm1-like* as an adipogenesis-induced gene in a report in 2008, wherein a role for Wdnm1-like protein in modulating MMP activity was also reported. Shortly after this publication in 2008, when considering generation and study of null mice for *Wdnm1-like*, it became apparent that the human locus had a single base pair deletion early in an otherwise predicted open reading frame. Therefore, it likely encoded a pseudogene in humans and not the Wdnm1-like protein. Given this, it was decided that this laboratory (Smas) would not go further on the project in respect to the

study of the Wdnm1-like protein. As such, the human gene or transcript has not been addressed in studies from this laboratory (Smas), and we not aware of any data reporting on expression of this transcript in human adipose tissue. However, while not the subject of such studies, murine Wdnm1-like has been mentioned within several publications on adipocytes and adipose tissue [4-6]. These relate to the finding that the Wdnm1-like transcript is highly enriched in expression in white *vs.* brown murine adipocytes/adipose tissue [4-6]. Of interest, knockout mice for Wfdc21 (Wfdc21$^{tm1a(KOMP)Wtsi}$) are now available by embryo resuscitation through the KOMP project (Project ID: CSD49368) and would serve as a highly useful system in which to address the role of Wdnm1-like in DC cell maturation in mice.

In the prior publication on Wdnm1-like from this laboratory (Smas), our studies utilized a murine Wdnm1-like expression construct with a C-terminal epitope tag, and cell transfection studies. These showed that a protein of predicted size for Wdnm1-like was ectopically expressed and secreted into culture media. Dijkstra and Ballingall do accurately describe our studies and clearly state that our work on Wdnm1-like utilized recombinant ectopic expression of the predicted murine Wdnm1-like open reading frame. However, one would have liked to have seen Dijkstra and Ballingall make it even more clear, earlier on in their text, that the endogenous Wdnm1-like protein has not yet been demonstrated in any system/species. A quick Google search fails to find an available antibody to the Wdnm1-like protein, so the endogenous protein remains to be investigated.

The work by Dijkstra and Ballingall makes several very important points:

1. It provides well-needed clarification and extensive documentation that apparently only for hominids does the locus for *Wdnm1-like* encode a long non-coding RNA, while in all other species specifically examined (which was quite extensive) this locus contains an open reading frame for the Wdnm1-like protein.

2. It raises concerns as to the ultimate responsibility of Wang and co-authors to report the full range of information on such distinctions within their *Science* report, if they indeed were aware of such. Only Wang and co-authors can inform us of their extent of knowledge of the protein coding nature of murine *vs.* human *Wdnm1-like* at the time of their *Science* publication. Thus it is not possible at this point to know whether such information was selectively omitted. But as Dijkstra and Ballingall point out, Wang and co-authors refer to the GenBank entry for murine Wdnm1-like , also known as 100001G20Rik and now formally named Wfdc21. This GenBank entry contains citations for publications on murine Wdnm1-like [1,7]. It seems very odd if Wang and co-authors were not aware of the distinctions between the human and murine forms of *Wdnm1-like*, particularly in today's age of well-curated databases. In fact, the NCBI Unigene entry for this gene reveals the human version is annotated as *Wdnm1-like* pseudogene (LOC645638, Hs.463652). Perhaps this was one more instance of a research group "rediscovering and renaming" a gene that was previously published on. This is all too common of late; doing so essentially tosses aside the already peer-reviewed and published work of others [1,7]. However, until further clarification is forthcoming by Wang and co-authors, one would hope they would be provided with the benefit of the doubt on the facts and intentions in regard to this matter.

3. A third valid concern raised by Dijkstra and Ballingall, is whether the knowledge that the murine gene is predicted to be protein encoding, while the human gene encodes a long non-coding RNA (*lnc-DC*), impacts the quality or interpretation of the data in the manuscript by Wang and co-authors. It appears that for the vast majority of the studies in their *Science* publication, Wang and co-authors utilized human cell culture systems, and limited studies were conducted in murine systems. Thus we are in agreement with Dijkstra and Ballingall that the essential conclusions of the *Science* report

are not dependent on the murine studies, or the fact that *Wdnm1-like* encodes a long non-coding RNA in humans and the Wdnm1-like protein in mice. However Wang and co-authors used studies in murine systems to further address whether knockdown of Wdnm1-like affected DC differentiation, finally claiming "that *lnc-DC* is vital for DC differentiation in both human and mice". This strikes one as a very disturbing claim as it implies that in mice this gene locus functions as a long non-coding RNA, when all the available evidence clearly argues against such. This leaves the readers of *Science* with less than the complete and indeed even an obfuscated picture of the *Wdnm1-like* gene. Readers, and one presumes also the reviewers of the *Science* report, were thus unable to fully judge the quality and relevance of the studies that Wang and co-authors conducted in murine systems. As indicated by Dijkstra and Ballingall, Wang and co-authors referring to or renaming murine *Wdnm1-like* as the "*mouse lnc-DC ortholog*", without also educating their readers on the protein coding nature of the murine gene, appears a serious disservice to their readers.

If Wang and co-authors were aware of the fact that murine *Wdnm1-like* gene was most likely protein-encoding, and whether this was the case or not is unknown at this juncture, they have indeed failed the readers of *Science* in this regard. One would hope to see some explanation from Wang and co-authors on this matter. The points raised by Dijkstra and Ballingall serve as "food for thought" for all of us on the responsibility of authors to fully inform their readers in regard to the state of current knowledge in respect to the scientific content of their manuscripts. It is left up to the readers of the paper by Dijkstra and Ballingall, the text of the *Science* report by Wang and co-authors and the comments furnished herein, to reach their individual opinions on this specific matter.

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We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: C. Smas: As a coauthor on prior work on Wdnm1-like, I might be regarded as a scientific competitor on this work. However we have not worked on this gene since 2008.

Author Response 27 Aug 2014

Johannes M. Dijkstra, Fujita Health University, Japan

Dear Dr. Smas and Dr. Ren,

Thank you for your extensive comments, which we embrace as generally positive. We are happy that you approve of how we summarized existing reports on *Wdnm1-like* in rodents. Furthermore, you added some nice insights and discussion points, and provided the readers with additional references that confirm that murine *Wdnm1-like* transcript is highly enriched in white vs. brown adipocytes/adipose tissue. You state correctly that endogenous Wdnm1-like protein has not been reported so far. However, a search of the PeptideAtlas database http://www.peptideatlas.org (Desiere, *et al*. 2006) which includes peptides identified by mass spectrometry from multiple species, identified a rat peptide encoded by correctly spliced *Wdnm1-like* . This suggests that the Wdnm1-like protein is present in the rat. We will include this information in the text after all the referee reports have been received. Whether the style of our manuscript is excessively critical is open to discussion, especially since both our research groups appear to share similar criticisms of the Wang et al. report. However, if you feel that a sentence is excessively critical please send us suggestions for changes (in a private mail?) and we will follow your lead.

Sincerely,

Johannes M. Dijkstra and Keith T. Ballingall

Reference

Desiere, et al., *"The PeptideAtlas Project"*, Nucleic Acids Research, 2006, 34, D655-D658

Competing Interests: No competing interests were disclosed.