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Metopic suture and *RUNX2*, a key transcription factor in osseous morphogenesis with possible important implications for human brain evolution

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Abstract

Background. Overall, the comparative data available on the timing of metopic suture closure in present-day and fossil members of human lineage, as well as great apes, seem to indicate that human brain evolution occurred within a complex network of fetopelvic constraints, which required modification of frontal neurocranial ossification patterns, involving delayed fusion of the metopic suture. It is very interesting that the recent sequencing of the Neanderthal genome has revealed signs of positive selection in the modern human variant of the *RUNX2* gene, which is known to affect metopic suture fusion in addition to being essential for osteoblast development and proper bone formation. It is possible that an evolutionary change in *RUNX2*, affecting aspects of the morphology of the upper body and cranium, was of importance in the origin of modern humans. Thus, to contribute to a better understanding of the molecular evolution of this gene probably implicated in human evolution, we performed a comparative bioinformatic analysis of the coding sequences of *RUNX2* in *Homo sapiens* and other non-human Primates.

Results. We found amino-acid sequence differences between *RUNX2* protein isoforms of *Homo sapiens* and the other Primates examined, that might have important implications for the timing of metopic suture closure.

Conclusions. Further studies are needed to clear the potential distinct developmental roles of different species-specific *RUNX2* N-terminal isoforms. Meantime, our bioinformatic analysis, regarding expression of the *RUNX2* gene in *Homo sapiens* and other non-human Primates, has provided a contribution to this important issue of human evolution.

Key words

RUNX2, metopic suture closure, human brain evolution.

Introduction

Cranial suture fusion patterns reflect very likely the phylogeny of primate taxa (Cray *et al.* 2008, 2010). In addition to being useful to deduce phylogenetic information, the analysis of variations of suture closure patterns may also provide a bet-

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ter understanding of functional and developmental implications of suture biology, including the role of genetics in the species studied.

Yet for research on human evolution, and human brain evolution in particular, probably it's most interesting the study of the timing of metopic (or medio-frontal) suture closure.

The metopic suture normally becomes obliterated later in modern humans (*Homo sapiens*) than in chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*) or gorilla (*Gorilla gorilla*). In fact, while in African great apes, the metopic suture normally fuses shortly after birth, in modern humans, instead, the metopic suture closes comparatively late, well after birth (Falk *et al.*, 2012). In concomitance with a late metopic suture closure, human infants and adults exhibit a relatively high proportion of partially fused and unfused metopic sutures, but this condition is infrequent in the great apes (Ashley-Montagu, 1937; Hauser and De Stefano, 1989; Eroğlu, 2008; Falk *et al.*, 2012).

Early metopic suture fusion, in effect, is a feature uniting crown anthropoid primates (Kay *et al.*, 2004). Thus, early fusion, as observed in the great apes, appears as the primitive state, and relatively late metopic suture fusion in modern humans appears as a derived state.

But when did this occur in the course of the hominin evolution ("hominins" refers to humans and our evolutionary ancestors back to the separation of the human and African ape lineages)?

The presence of a still patent fontanelle and of a partially fused metopic suture, as reproduced by its natural brain endocast, in the so-called Taung child (*Australopithecus africanus*; see Dart, 1925) (Hrdlička, 1925), aged 3-4 years at death (Lacruz *et al.*, 2005) and lived between about 3.0 and 2.5 million years ago (Holloway *et al.*, 2004), in addition to the relatively high incidence of partially fused and unfused metopic sutures in gracile fossil subadult and adult hominins (Falk *et al.*, 2012) that lived between about 2.0 and 1.5 million years ago (Holloway *et al.*, 2004) (the term "gracile hominins" is used here to indicate fossils attributed to the genus *Australopithecus* and early *Homo*, as opposed to *Paranthropus*, to which so-called "robust hominins" are instead attributed), seems to indicate that the modern human-like pattern of late metopic fusion may have become adaptive during early hominin evolution. Furthermore, comparatively high frequencies of partially fused or unfused metopic sutures in *Homo erectus* from Asia and in the Neanderthals (*Homo neanderthalensis*) seem to show that the trend toward late metopic suture fusion continued in mid-to-late Pleistocene hominins (Falk *et al.*, 2012).

Therefore, although some authors regard variation in metopic suture fusion as an adaptively neutral feature (Ashley-Montagu, 1937; Eroğlu, 2008), comparative data gathered on the timing of metopic suture closure in fossil hominins, modern humans and great apes (Falk *et al.*, 2012) support the hypothesis that hominin brain evolution occurred within a complex network of fetopelvic constraints, which required modification of frontal neurocranial ossification patterns.

Selective pressures favoring delayed fusion might thus have resulted from three different, but mutually nonexclusive, aspects of perinatal ontogeny: first, the difficulty of giving birth to large-headed neonates through birth canals that were reconfigured for bipedalism (the so-termed "obstetric dilemma"); second, high early postnatal brain growth rates; and third, reorganization and expansion of the prefrontal neocortex, parts of which are differentially enlarged in humans (Semendeferi *et al.*, 2001) and known to

be crucial for our advanced cognitive capabilities (Wood and Grafman, 2003).

During hominin evolution, when the obstetric dilemma arose, bipedalism was refined in conjunction with an evolutionary increase in neonate and adult brain sizes, and the morphology of the birth canal constrained the size and shape of the neonate (DeSilva and Lesnik, 2006; Berge and Goularas, 2010; DeSilva, 2011). It's possible that already in early hominins, increased mobility of the neurocranial bones through delayed metopic suture fusion might have represented an adaptive advantage facilitating birth (Tague and Lovejoy, 1986).

Moreover, taking into account that compared with chimpanzees, human brains continue to grow at high fetal-like rates throughout the first postnatal year of life (Leigh, 2004), it is also reasonable to hypothesize that relatively late metopic suture closure in modern humans reflects an evolutionary adaptation of the growing frontal neurocranium to keep up with high brain growth rates (Falk *et al.*, 2012).

Finally, in addition to reflecting an adaptation to high postnatal brain growth rates, a late metopic suture closure may also have been associated with the evolution of certain morphological (Falk *et al.*, 2000) and cytoarchitectural features (Semendeferi *et al.*, 2011) of the prefrontal neocortex in at least some species of gracile early hominins (Falk *et al.*, 2012).

Although new evidence has only recently been found in fossil hominins, already in 1941 Weidenreich linked hypothetically persistence of the metopic suture to the evolutionary developmental transformation of the human skull, including neurocranial expansion and thinning of the vault bones. Hypotheses on the genetic control of metopic suture fusion have also been proposed for a long time (Torgersen, 1951). In more recent years, investigating what causes craniosynostosis (the premature fusion of calvarial sutures), the sutural biology research has shed some light on the processes of normal neurocranial suture fusion. The current genetic evidence indicates that a complex molecular network of at least 10 key genes mediates neurocranial suture fusion, and that these genes act differently on different sutures and in distinct species (Coussens *et al.*, 2007). It is very interesting that the recent sequencing of the Neanderthal genome reveals signs of positive selection in the modern human variant of one of these key genes, *RUNX2* (also termed *CBFA1*), which is known to affect metopic suture fusion (Green *et al.*, 2010).

RUNX2 is one of three mammalian paralogous genes that encode proteins homologous to *Drosophila* Runt, and that are crucial for proper embryonic development. But the essential functions of *RUNX1* and *RUNX3* during embryogenesis are manifested in non-osseous tissues (Miyoshi *et al.*, 1991; Okada *et al.*, 1998; Miyamoto *et al.*, 2000; Levanon *et al.*, 2002; Li *et al.*, 2002), although these *RUNX* genes also contribute to endochondral bone formation (Lian *et al.*, 2003; Brenner *et al.*, 2004; Yoshida *et al.*, 2004). Only *RUNX2* is really essential for osteoblast development and proper bone formation (Schroeder *et al.*, 2005). This gene, that is located in humans on chromosome 6 (6p21) and spans about 220 kb (Levanon *et al.*, 1994), encodes Runt-related transcription factor 2 (RUNX2) also known as core-binding factor subunit alpha-1 (CBF-alpha-1), a member of the Runt domain family of transcription factors. This protein binds, by its highly conserved 128-amino acid Runt homology domain, a specific consensus DNA sequence to regulate transcription of numerous genes and thereby control osteoblast development from mesenchymal stem cells and maturation into osteocytes.

There are multiple RUNX2 protein isoforms, which differ from each other in certain domains and probably in specialized functions (Schroeder *et al.*, 2005). The *RUNX2* gene, in fact, in mammalian genomes encodes several transcripts, which are derived from two different promoters and alternative splicing. The differential usage of the promoters along with various processes of alternative splicing generates distinct isoforms (Levanon and Groner, 2004; Terry *et al.*, 2004; Stock and Otto, 2005). The promoters, P1 (distal) and P2 (proximal), are separated by exon I and a large intron and drive the expression of the two major isoforms of the RUNX2 protein, respectively, type II (also commonly called Cbfα1/p57, Cbfα1/iso or til-1, and here named in humans isoform *a*) and type I (also generally called Cbfα1/p56, Cbfa1/org or PEBP2αA, and here named in humans isoform *c*), which begin with two alternative amino-terminal sequences (namely MASNS and MRIPV). Moreover, this double promoter structure is conserved in human and murine *RUNX2* genes, as well as the other mammalian paralogous *RUNX* genes, *RUNX1* and *RUNX3* (Levanon and Groner, 2004), along with what seems to be another common feature of the Runt-related genes, namely the possibility of expression of further protein isoforms resulting from alternative splicing (Bae *et al.*, 1994; Levanon *et al.*, 1996; Tsuji and Noda, 2000).

In short, the osseous cells, in the course of their differentiation, may express a complex array of RUNX2 proteins, which overall contribute to regulate gene transcription and share the same functional domains like polyglutamine and polyalanine domain (QA), DNA-binding Runt homology domain (RHD), nuclear localization signal (NLS), proline-serine-threonine rich region (PST), nuclear matrix targeting signal (NMTS) and the TLE/groucho interacting carboxyterminal pentapeptide VWRPY (Lindenmuth *et al.*, 1997; Imai *et al.*, 1998; Thirunavukkarasu *et al.*, 1998; Quack *et al.*, 1999; Javed *et al.*, 2000; Zaidi *et al.*, 2001; Stock and Otto, 2005; Schroeder *et al.*, 2005).

Although necessary for gene transcription and osteoblast development, RUNX2 alone is not, however, sufficient for optimal gene expression or bone formation. In fact, several studies have indicated that RUNX2 is a context-dependent transcriptional activator and repressor (Li and Xiao, 2007) that interacts with a large cohort of other regulatory proteins, suggesting a complex mechanism of osteoblastogenesis control by this factor. It, receiving input from extracellular signals (such as, for example, bone morphogenetic proteins, transforming growth factors-β, fibroblast growth factors, parathyroid hormone, vitamin D_3) and intracellular proteins (such as Msx2, Dlx5, Twists, etc.) to promote or suppress the expression of other genes involved in bone formation, is a central organizing hub for transcriptional regulation in mesenchymal precursors and osteoblasts.

Moreover, RUNX2 also appears to be necessary for normal metopic suture morphogenesis and the regulation of suture patency (Cray *et al.*, 2008). In effect, *RUNX2* is the only gene in the human genome known to cause cleidocranial dysplasia, a rare autosomal dominant disease, which is characterized by delayed metopic suture fusion and pathologies, such as extreme bulging of the forehead, hypertelorism, hypoplastic or aplastic clavicles, a bell-shaped rib cage, and dental abnormalities (Mundlos *et al.*, 1997; Bufalino *et al.*, 2012). Some of these features affect morphological traits for which modern humans differ from Neandertals as well as other earlier hominins. It may therefore be reasonable to hypothesize that an evolutionary change in *RUNX2*, affecting aspects of the morphology of the upper body and cranium, was of importance in the evolution of modern humans (Green *et al.*, 2010). In effect, the morphological

differences between modern humans, earlier hominins and the great apes are, most likely, the product of evolutionary changes during development (Carroll, 2003), as it is indicated, for example, by the findings of various comparative studies of skull ontogeny in *Homo sapiens*, chimpanzees and fossil hominins (Dean *et al.*, 2001; Ponce de León and Zollikofer, 2001; Lieberman *et al.*, 2002; Penin *et al.*, 2002; Williams *et al.*, 2002).

Consequently it is important to investigate the molecular evolution of *RUNX2*, a key gene in skeletal development and the regulation of metopic suture closure, whose potential evolutionary change may be related to modification of frontal neurocranial ossification patterns, occurred during hominin brain evolution.

Therefore, with the aim to increase knowledge about the origin of modern humans, the goal of the present research was to integrate the previous morphological and genetic findings, concerning evolutionary changes of cranial morphogenesis processes relevant to hominin evolution, with data obtained from an bioinformatic analysis regarding expression of the *RUNX2* gene in *Homo sapiens* and other non-human Primates. By comparative analysis of the coding sequences of *RUNX2* in modern humans and our closest living evolutionary relatives, and through the study of homologies and divergences, we wanted to contribute to a better understanding of the molecular evolution of this key transcription factor with important roles in skeletal development, and probably implicated in human evolution.

Materials and methods

Bioinformatic analysis was carried out on the nucleotide and amino-acid sequences of the main RUNX2 isoforms in *Homo sapiens,* comparing them to corresponding sequences in four other species of Primates, *i.e.*, *Pan troglodytes* (chimpanzee), *Gorilla gorilla* (gorilla), *Pongo abelii* (Sumatran orangutan), and *Macaca mulatta* (rhesus macaque).

With regard to nucleotide alignments in this phase of the study, we restricted our analysis to only the coding regions of the *RUNX2* gene. By comparing the coding sequences we aimed to detect the presence of homologies and divergences among the proteins of the five species examined.

With regard to the amino-acid alignments, two parameters have been taken into account: sequence identity, *i.e.*, the proportion or percentage of identical residues paired after the alignment, and sequence similarity, *i.e.*, the proportion or percentage of residues with similar chemical-physical characteristics, paired after alignment. These parameters have been evaluated, by using *Protein Blast programme* (*http:// www. ncbi.nlm.nih.gov*), because the analysis of them provides a general overview of the phylogenetic distances among the Primate species examined.

Furthermore, we also proceeded to analyze both the absolute (over the entire coded sequence) and relative (to the level of each translated exon) frequencies regarding the amino-acid cysteine, because it plays an important role in the structure of proteins. The protein regions, containing more cysteine residues, may probably be most involved in significant structural and functional variations in coincidence of any change in amino-acid sequence.

Information concerning the *RUNX2* gene was obtained from the database at the University of California, Santa Cruz (UCSC, *http://genome.ucsc.edu*), referring to the

latest published version of the human genome (*http://hgdownload.cse.ucsc.edu/goldenPath/hg18*).

In particular, we used the *refGene* table, containing all gene sequences coding and non-coding for proteins. In this way, it was possible to obtain detailed information on human genes, such as: chromosome, position of the start and the end of transcription, position of the start and the end of coding part, the number and the positions of exons.

The annotations of the orthologous genes, relating to the other four species examined, were obtained using the *LiftOver* algorithm (*http://genome.ucsc.edu/cgi-bin/hgLiftOver*).

This tool allows one to convert genomic coordinates and genomic annotations between different versions of the genomes in the UCSC database. By so doing, on the basis of the position of the *RUNX2* gene on the human genome, it was possible to derive the corresponding coordinates on the other five genomes. Considering their positions, all available nucleotide sequences of the various transcripts encoded by the *RUNX2* gene in the genomes of the species examined were downloaded from the database, and were aligned using the *ClustalW* algorithm. From the analysis of each alignment concerning either the transcripts derived from P1 promoter or the transcripts derived from P2 promoter, we aimed to obtain the positions of any differences in coding nucleotide sequences among the five species. The coordinates of the start and the end of the exons on the human genome were mapped on each alignment, in order to further our analysis.

Once the amino-acid sequences were aligned using *ClustalW*, the columns of residues were taken into consideration. Any residue lined-up is to be considered implicitly related to evolution.

For reasons of space, we have included only two figures of details of the amino-acid alignments concerning the RUNX2 protein isoforms in the species examined, in addition to two tables in which we have listed data obtained about sequence identity and sequence similarity.

Results

First of all, in *Homo sapiens* and macaques there are two isoforms which are derived from the distal P1 promoter (Fig. 1), as well as one (in humans) or two (in macaques) isoforms which are derived from the proximal P2 promoter (Fig. 2), whereas in chimpanzees and gorillas there are only two P1 promoter-derived isoforms (Fig. 1) and in orangutans there are three isoforms which are uniquely derived from the proximal P2 promoter (Fig. 2).

That said, the results of the analysis of the sequence identity and sequence similarity parameters were the following:

- The chimpanzee, gorilla and macaque full-length P1-derived isoforms compared with the human isoform *a* showed both sequence identity and sequence similarity of 99 percent (Table 1);
- The orangutan full-length P2-derived isoform compared with the human isoform *c* showed both sequence identity and sequence similarity of 100 percent (Table 2);
- The macaque full-length P2-derived isoform compared with the human isoform *c* showed both sequence identity and sequence similarity of 99 percent (Table 2).

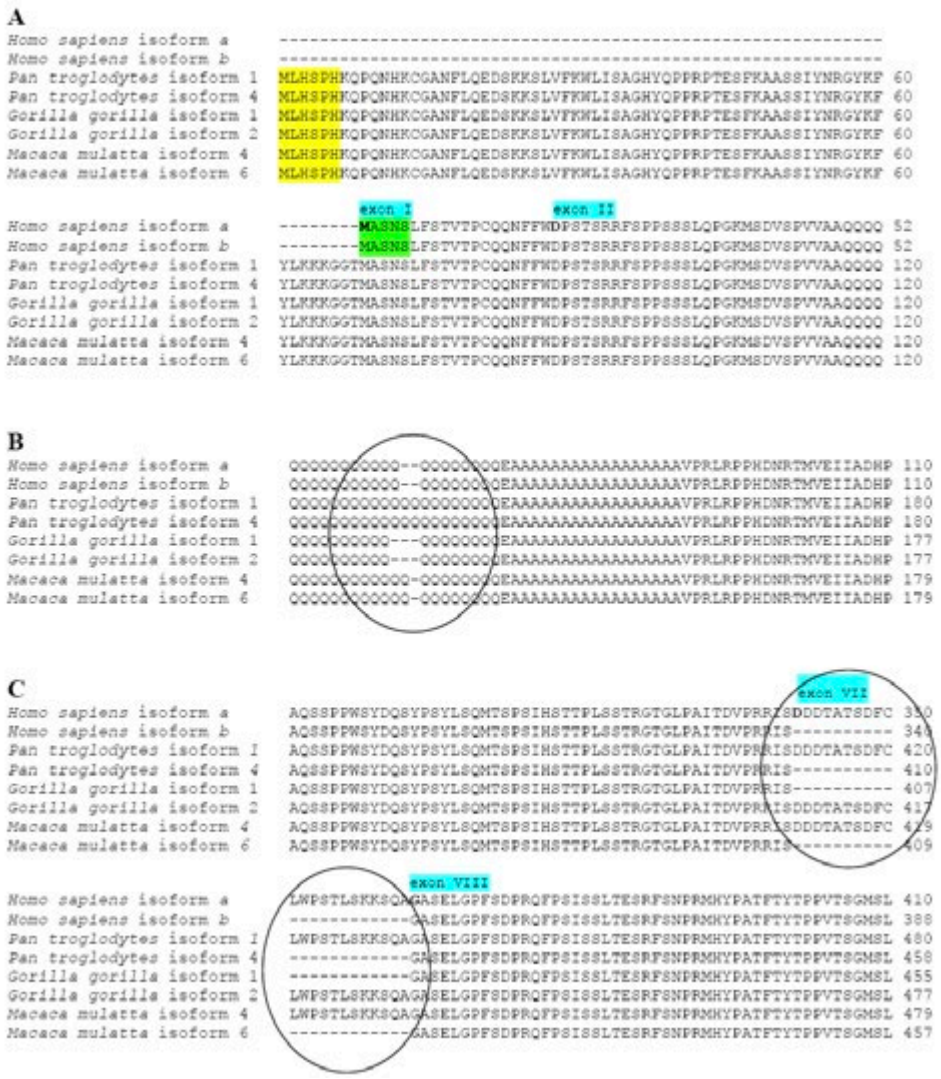

Figure 1 – Details of the amino-acid alignment concerning the P1 promoter-derived RUNX2 isoforms of *Homo sapiens* (modern human), *Pan troglodytes* (chimpanzee), *Gorilla gorilla* (gorilla), and *Macaca mulatta* (Rhesus macaque). A - The amino-termini of the proteins. The start sequence is highlighted in yellow in the non-human primate proteins, while it is highlighted in green in the human proteins. B - The polyglutamine and polyalanine (QA) domains of the proteins: the sites having amino-acid sequence differences are shown in the circle. C - The protein region (shown in the circles) encoded by *RUNX2* exon VII: alternative splicing of this exon generates more protein products.

Therefore, overall, our analysis showed mostly the presence of conserved sequences between the various RUNX2 isoforms of *Homo sapiens* and the other Primates examined. However, there are two significant amino-acid sequence differenc-

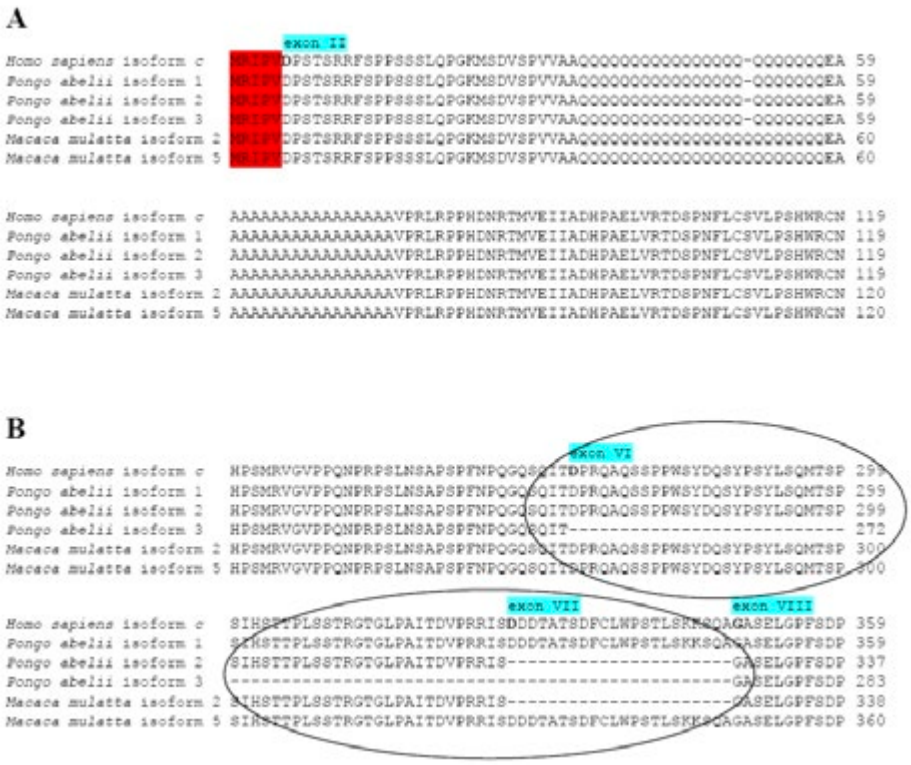

Figure 2 – Details of the amino-acid alignment concerning the P2 promoter-derived RUNX2 isoforms of *Homo sapiens* (modern human), *Pongo abelii* (Sumatran orangutan), and *Macaca mulatta* (Rhesus macaque). A - The amino-terminal regions of the proteins. The start sequence is highlighted in red. B - The protein region (shown in the circles) generated by alternative splicing.

es between the human RUNX2 isoforms derived from the distal P1 gene promoter (namely *a* and *b*) and those of other analyzed species. In fact, the African ape RUNX2 isoforms, as well as the corresponding macaque N-terminal isoforms which are derived from the P1 promoter, appear, compared to the human isoforms *a* and *b*, with additional 68 N-terminal amino-acid residues starting with the sequence MLHSPH instead of MASNS (Fig. 1A); furthermore, in the polyglutamine and polyalanine (QA) domain, located near the protein amino-terminus, the human isoforms *a* and *b* are 2 glutamine residues shorter than the chimpanzee isoforms, which also appears to be longer than the gorilla isoforms (for three glutamine residues) as well as the corresponding macaque N-terminal isoforms (for one glutamine residue) (Fig. 1B).

Finally, the results of the cysteine residue distribution analysis in amino-acid sequences of the main RUNX2 isoforms of the species examined were the following:

- While the human P1-derived isoform *a* contains 5 cysteine residues in total, having 1 residue encoded in each of exons I, VII and VIII, and 2 residues in exon II, the human P1-derived isoform *b*, a splice product lacking exon VII, contains

Table 1 – Summary of the parameters of sequence identity (Id) and sequence similarity (Pos) concerning the full-length P1 promoter-derived RUNX2 isoforms of *Homo sapiens* (modern human), *Pan troglodytes* (chimpanzee), *Gorilla gorilla* (gorilla), and *Macaca mulatta* (Rhesus macaque). Also shown are the amino-acid sequence lengths and the proportion or percentage of gaps that are within the alignment relatively to the human sequence.

Species/isoform	Id (%)	Lenght (aa)	Pos (%)	Gaps
Homo sapiens isoform *a*	521/521 (100%)	521	521/521 (100%)	0/521 (0%)
Pan troglodytes isoform 1	518/523(99%)	591	519/523(99%)	2/523(0%)
Gorilla gorilla isoform 2	518/521(99%)	588	519/521(99%)	1/521(0%)
Macaca mulatta isoform 4	521/522(99%)	590	521/522(99%)	1/522(0%)

Table 2 – Summary of the parameters of sequence identity (Id) and sequence similarity (Pos) concerning the full-length P2 promoter-derived RUNX2 isoforms of *Homo sapiens* (modern human), *Pongo abelii* (Sumatran orangutan), and *Macaca mulatta* (Rhesus macaque). Also shown are the amino-acid sequence lengths and the proportion or percentage of gaps that are within the alignment relatively to the human sequence.

Species/isoform	Id (%)	Lenght (aa)	Pos (%)	Gaps
Homo sapiens isoform *c*	507/507 (100%)	507	507/507 (100%)	0/507 (0%)
Pongo abelii isoform 1	507/507(100%)	507	507/507 (100%)	0/507 (0%)
Macaca mulatta isoform 5	507/508(99%)	508	507/508(99%)	1/508(0%)

4 cysteine residues in total. Each chimpanzee, gorilla and macaque full-length P1-derived isoform contains instead 6 residues overall, having 2 residues encoded in both exons I and II, in addition to 1 residue in both exons VII and VIII, while each chimpanzee, gorilla and macaque P1-derived isoform lacking the region encoded by exon VII contains 5 residues in total.

- The human isoform *c*, whose expression is driven by the P2 promoter, contains 4 cysteine residues in total, having 1 residue encoded in both exons VII and VIII, and 2 residues in exon II. Compared to human isoform *c*, the orangutan and macaque full-length P2-derived isoforms contain the same number and distribution of cysteine residues, while the other orangutan and macaque P2-derived isoforms, generated by alternative splicing, contain 1 cysteine residue less.

We may therefore assume that the regions most affecting the protein function are probably encoded in exons I, II, VII and VIII. Overall, however, the presence of few cysteine residues is consistent with possible frequent conformational changes of this transcription factor that interacts with other numerous regulatory proteins.

Discussion

The protein regions, in which significant differences appear between human and non-human Primate RUNX2 isoforms (all derived from the distal P1 promoter), *i.e.*, the amino-terminus of the RUNX2 protein and its glutamine-alanine (QA) rich domain, as well as the proline-serine-threonine (PST)-rich region, are activation

domains that contribute to the transcriptional activity of RUNX2 (Schroeder *et al.*, 2005).

The functional relevance of the polyglutamine and polyalanine (QA) domain is shown by the fact that this region is frequently altered by genetic mutations in cleidocranial dysplasia families (Otto *et al.*, 2002), and polymorphic expansions or contractions of the QA domain may also influence skeletal strength and morphology in humans and other species (Vaughan *et al.*, 2002; Fondon and Garner, 2004).

Therefore, it is reasonable to assume that the sequence differences described above in the QA domain of RUNX2 isoforms between humans and non-human Primates may reflect significant functional differences of the transcription factor in different species.

The amino-terminus of RUNX2 is also probably very important for regulatory functions, and not only because it may be necessary for maximal transcriptional activation. In effect, in addition to its activation potential, the amino-terminus of RUNX2 has repressive activity and can inhibit DNA binding (Inman *et al.*, 2005; Schroeder *et al.*, 2005), so it may be a context-dependent regulator of RUNX2 activity.

Moreover, although the mechanisms for the isoform-specific developmental roles are not yet clear, however they may include preferential promoter induction, different translational efficiencies and/or binding of other proteins to the alternative amino-termini of the different RUNX2 isoforms (Schroeder *et al.*, 2005; Li and Xiao, 2007). On the other hand, it seems likely that different RUNX2 isoforms, due to possible distinct developmental roles, may be capable of interacting with unique sets of regulatory cofactors that influence their function, or be transcriptionally regulated differently (Schroeder *et al.*, 2005). So in *Homo sapiens* there are two major isoforms, with alternative amino-termini, that are thought to have separate functions in skeletogenesis (Li and Xiao, 2007), and that show to be differently affected by at least certain regulatory proteins (Banerjee *et al.*, 2001, Schroeder *et al.*, 2005).

In great apes, instead, there is only one type of N-terminal isoform (or at least it is largely predominant), deriving or from the distal P1 promoter, notably in chimpanzee and gorilla which are our closest living evolutionary relatives, or from the proximal P2 promoter, *i.e.*, in orangutan whose lineage diverged from that of *Pan*, *Gorilla* and *Homo* around 14-15 million years ago (Carroll, 2003).

It is noteworthy that a P1 promoter-derived isoform with additional 68 N-terminal amino-acid residues starting with the sequence MLHSPH also exists in mice in addition to the two major amino-terminal isoforms (*i.e.*, type I and type II) that are present in modern humans as well. This third major amino-terminal isoform (type III, also called Osf2/Cbfa1, Runx2/osf2 or Cbfa1/Osf2), described by Ducy *et al.* (1997), is derived in the mouse from an alternative translation start in exon I and has a different transactivation potential compared with RUNX2-type I (Li and Xiao, 2007). Yet, the sequence responsible for this additional N-terminal 68-amino-acid segment beginning with MLHSPH is not conserved as a coding region in the *Homo sapiens RUNX2* gene (Lee *et al.*,1997; Xiao *et al.*, 1998; Stock and Otto, 2005; Schroeder *et al.*, 2005); in fact, this sequence is a part of 5′-untranslated regions (5′-UTRs) in both human P1-derived transcripts. But clearly it is not so in chimpanzees, gorillas and macaques.

Thus, a reasonable hypothesis is that the differences described above between N-terminal isoforms of *Homo sapiens* and non-human Primates might reflect distinct patterns of regulation of osteoblast development and bone formation in modern

humans respect to our closest living evolutionary relatives, and in particular might have important implications for the timing of metopic suture closure. If so, the late metopic suture closure in modern humans in comparison with the great apes (Falk *et al.*, 2012) might have a plausible explanation at the level of the molecular control of calvarial suture fusion.

However, further studies are needed. Because the significant amino-acid sequence differences between the orthologous RUNX2 proteins of *Homo sapiens* and other Primates are in the P1 promoter-derived isoforms, it is clearly important to better understand the potential functional differences between the distinct N-terminal RUNX2 isoforms possibly imparted by their different amino-termini (Li and Xiao, 2007), both within the same and among different species.

Several studies have already provided some evidence about the influence on bone formation of likely differences in function between the two major RUNX2 isoforms (*i.e.*, the P2 promoter-derived type I isoform and the P1 promoter-derived type II isoform), although the specific molecular mechanisms involved in the cellular- and tissue-specific distribution and potential functions of the two isoforms still need to be further investigated (Choi *et al.*, 2002; Xiao *et al.*, 2004, 2005; Li and Xiao, 2007).

It is generally accepted that RUNX2 type II represents a highly regulated isoform intensely expressed in mature osteoblasts and terminally differentiated hypertrophic chondrocytes, while RUNX2 type I, sufficient for early osteoblastogenesis, is thought to be a more broadly expressed isoform, constitutively present also in earlier precursors of osteoblasts and chondrocytes, and even in non-osseous mesenchymal cells (Stock and Otto, 2005; Schroeder *et al.*, 2005; Li and Xiao, 2007). Furthermore, a study that investigated RUNX2 isoform expression in cranial suture morphogenesis found that while type I isoform was most intensely expressed in the sutural mesenchyme, type II isoform was predominantly expressed in the osteogenic fronts of the calvaria (Park *et al.*, 2001).

In short, it is possible that the multiple signaling pathways and the numerous regulatory cofactors modulating *RUNX2* gene expression, the activity of the protein products and the subsequent bone formation may differently regulate the distinct N-terminal isoforms in the same species as well as in different species. On the other hand, because RUNX2 is a transcription factor, changes in *RUNX2* expression could be of functional and evolutionary significance, being able to contribute to a potential divergence in the role of RUNX2 between humans and apes.

Conclusions

The present bioinformatic analysis on the expression of the *RUNX2* gene in *Homo sapiens* and other non-human Primates is a contribution to recognize changes in the molecular mechanisms underlying frontal neurocranial ossification patterns during hominin brain evolution. Future research on the roles of different species-specific N-terminal isoforms of RUNX2 during morphogenesis and differentiation may lead to a better understanding of the developmental and functional aspects of those changes. Further studies of RUNX2 in higher Primates, the closest living evolutionary relatives of present-day humans, may also be useful to expand knowledge on skeletal diseases, in addition to that on human evolution.

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