Chapter 5.2


(Colour images from this chapter can be seen in the appendix)
Genetic heterogeneity in Rubinstein-Taybi syndrome: mutations in the CBP and EP300 gene are both disease causing.

Jeroen H Roelfsema1, Stefan J White1, Yavuz Arıyürek1, Deborah Bartholdi3, Dunja Niedrist3, Francesco Papadia4, Carlos Bacino5, Johan T den Dunnen1, Gert-Jan B van Ommen1, Martijn H Breuning1, Raoul C Hennekam2 and Dorien JM Peters1

1Center for Human and Clinical Genetics, LUMC, Sylvius Laboratory, Wassenaarseweg 72, 2333AL, Leiden, The Netherlands.
2Institute of Medical Genetics, University of Zurich, Switzerland.
3Department of Metabolic Diseases and Clinical Genetics, Pediatric Hospital Giovanni XXIII, Bari, Italy.
4Department of Clinical Genetics, Academic Medical Center, Amsterdam, The Netherlands.
5Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, United States of America.

Abstract

CREB Binding Protein and p300 function as transcriptional coactivators in the regulation of gene expression through various signal transduction pathways. Both are potent Histone Acetyl Transferases. The level of CREB Binding Protein is essential for normal development, as inactivation of one allele causes Rubinstein-Taybi syndrome. There is a direct link between loss of acetyl transferase activity and Rubinstein-Taybi syndrome, which indicates that the disorder is caused by aberrant chromatin regulation. We screened the entire CBP gene for mutations in Rubinstein-Taybi syndrome patients using methods to find point mutations and larger rearrangements. In 92 patients we were able to identify a total of 36 mutations in the CBP gene. Using Multiple Ligation-dependent Probe Amplification we not only found several deletions but also the first duplication in a Rubinstein-Taybi syndrome patient. We extended the search for mutations to the EP300 gene and showed that mutations in EP300 also cause this disorder. These are the first mutations identified in EP300 in a congenital disorder.

Introduction

Rubinstein-Taybi syndrome (RSTS) is a congenital disorder characterized by mental and growth retardation and a wide range of typical dysmorphic features. Facial dysmorphology includes down slanted palpebral fissures, broad nasal bridge, a beaked nose and micrognathia. Particularly noticeable are the broad thumbs and broad big toes. In addition, RSTS patients have an increased risk for tumor formation. Although various types of tumors have been described, there is an excess of tumors arising from developmental defects and tumors of brain or neural crest cell
derived tissue (Miller and Rubinstein 1995). Mutations in the gene coding for the CREB binding protein (CREBBP, also known as CBP), located on chromosome 16p13.3, were found to be responsible for causing the disorder (Petrij et al. 1995).

CBP serves as a transcriptional coactivator (Kwok et al. 1994). It has a transactivation domain but does not specifically bind to DNA. The name of the protein is based on the interaction with the CRE binding protein (CREB); however, CBP interacts with a large number of transcription factors. It is thought that CBP acts as an integrator of the signals from various pathways (Goodman and Smolik 2000). Transcription factors downstream from these pathways need to compete with each other for the limited amount of CBP available in the nucleus. The protein forms a physical bridge between the DNA binding transcription factors and the RNA polymerase II complex. In addition, CBP has intrinsic histone acetyl transferase (HAT) activity (Bannister and Kouzarides 1996). By acetylating histones it opens the chromatin structure at the locus that needs to be expressed, a process essential for gene expression. CBP is also capable of acetylating a large number of other proteins, for example the transcription factor p53 (Gu and Roeder 1997).

RSTS is considered to be an autosomal dominant disorder, however patients very rarely have children. Almost all mutations, therefore, occur de novo. The mutations found in patients range from relatively large microdeletions, removing the gene entirely, to point mutations. In addition, five translocations and two inversions disrupting the gene have been reported (Petrij et al. 2000). The microdeletions that remove the entire gene indicate that haploinsufficiency is the ultimate cause of the syndrome. Presumably, at critical moments during development the amount of CBP drops below a certain threshold because of the loss of one allele. How this loss of one allele actually causes the particular symptoms of RSTS, however, is unclear. Nevertheless, we know from patients with missense mutations and splice site mutations affecting only the HAT domain of CBP, that loss of HAT activity is sufficient to cause the syndrome (Murata et al. 2001; Kalkhoven et al. 2003).

In order to elucidate the complete spectrum of mutations we screened 92 RSTS patients for point mutations, small deletions or insertions and for larger deletions and duplications. Because we could not find mutations in the CBP gene in the majority of our patients we assumed that the remaining patients have mutations in other genes.

CBP shares homology with another protein, p300, encoded by the EP300 gene on chromosome 22q13.2 (Lundblad et al. 1995). Both proteins are particularly homologous at their binding sites for transcription factors and p300 also has a HAT domain. Like CBP it serves as a transcriptional coactivator. A likely candidate to screen, therefore, is EP300 and, indeed, we found three
mutations. These are the first mutations described in EP300 in a congenital disorder and they also prove that RSTS is a genetically heterogeneous disorder.

Material and Methods

The majority of the DNA samples described in this study were sent to us by clinicians in the Netherlands and many other countries as soluble genomic DNA from patients with a clinical diagnosis of RSTS. DNA from the rest of the patients was isolated from peripheral blood in our laboratory using standard protocols.

DGGE
DGGE was performed with a GC-clamp on either the forward or the reverse primer. Primers were selected to anneal to the flanking intron sequences in order to screen the splice sites and the branch sites, and were chosen using either WINMELT (Biorad) or MELT-INGENY (Ingeny B.V.) software. All oligonucleotides were synthesized by Sigma-Aldrich. Amplified fragments were analyzed on 9% polyacrylamide gels (37.5:1) with various linear denaturing gradients, optimized for each fragment, on the DCode system from Biorad. Gels were run at 90V at a constant temperature of 60ºC. An acrylamide mixture with 40% formamide and 7M urea was defined as 100% denaturant and acrylamide without these denaturing agents was defined as 0% denaturant.

SSCP
Electrophoresis was performed at room temperature using two types of gels. The first type was a polyacrylamide gel (49:1) with 1*TBE without glycerol and the second type was 0.5*MDE (National Diagnostics, Atlanta, Georgia) with 0.6*TBE and 10% glycerol. During amplification the fragments for SSCP analysis were radioactively labeled either by incorporation of $\alpha^{32}$P-dCTP or by using primers that were kinated using $\gamma^{32}$P-dATP (Amersham). Visualization of the fragments was done using the PhosphorImager (Molecular Dynamics).

MLPA
Probes were designed for 20 exons of the CBP and EP300 genes. MLPA was performed as described in (White et al. 2004). All samples were tested at least twice.

Sequencing and restriction digestions.
Sequencing was performed on the ABI 3700 from Applied Biosystems using the manufacturers standard protocol and reagents. Restriction digestions were performed according to the instructions of the manufacturer. Digestions or second sequencing reactions to confirm the first result were done on PCR fragments generated in an independent reaction. The deletion of 8
nucleotides in patient 256-1 was confirmed by PCR with an allele-specific primer, `tctccatctactagtagtg`, that skips the deleted part and anneals with 2 nucleotides after the deletion. The reverse primer has the sequence `gtctaaccaatcaaacag`.

Results

**Point mutations and small deletions or insertions in the CBP gene**

We screened the entire *CBP* gene for point mutations and small deletions or insertions using primarily DGGE, with target sequences that were not suited for DGGE being screened by SSCP analysis. The complete coding sequence and splice sites of the *CBP* gene required a total of 49 fragments of which 40 were screened using DGGE, approximately 83% of the coding sequence. Direct sequencing was used to identify the mutation after aberrant bands were found on DGGE or SSCP gels. All mutations were confirmed either by digestion with restriction enzymes when a restriction enzyme site was altered or by a second sequence analysis.

In 92 patients we found a total of 27 mutations (see table 1). The majority is predicted to lead to a premature translation stop but we also detected 5 putative missense mutations. Base substitutions leading to a premature stop codon or deletions and insertions leading to frame shifts can be clearly identified as disease causing mutations. A change of amino acids is much less clear, however, RSTS patients as a rule have *de novo* mutations. Since we were able to confirm the mutation as *de novo* for three of the mutations we consider them most likely to be disease causing. We do not have parental DNA of patients 228-1 and 260-1. All putative missense mutations are at the highly conserved HAT domain of CBP and the amino acids that are changed have residues that are conserved in both the mouse and the fruit fly (see fig.1). Unless we have an RNA sample from a patient we cannot check whether a splice site mutation actually leads to aberrant splicing. These mutations however, should also comply with the rule that mutations in RSTS patients occurred *de novo*. Except for the mutation in patient 39-1 for which parental DNA was not available, we could confirm the mutations that way. The mutation, a G to A, in the splice donor site flanking exon 24 in patient 39-1, however, is at the first position, which should in all splice donor sites, without exception, be a guanine. The splice site mutation of patient 211-1 could be analyzed on RNA isolated from a cell line. Subsequent sequence analysis proved that the mutation in the splice acceptor site flanking exon 22 leads to a deletion of exon 22 in the processed mRNA (Kalkhoven et al. 2003).
### Mutations in the CBP gene

<table>
<thead>
<tr>
<th>Individual</th>
<th>Exon</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>7-1</td>
<td>Exon 2</td>
<td>c.304 C&gt;T</td>
</tr>
<tr>
<td>177-1</td>
<td>Exon 5</td>
<td>c.1237 C&gt;T</td>
</tr>
<tr>
<td>212-1 *</td>
<td>Exon 28</td>
<td>c.4669 C&gt;T</td>
</tr>
<tr>
<td>27-1</td>
<td>Exon 29</td>
<td>c.4879 A&gt;T</td>
</tr>
<tr>
<td>2-1</td>
<td>Exon 31</td>
<td>c.6010 C&gt;T</td>
</tr>
<tr>
<td>16-1</td>
<td>Exon 31</td>
<td>c.6133 C&gt;T</td>
</tr>
<tr>
<td>178-3</td>
<td>Exon 31</td>
<td>c.6283 C&gt;T</td>
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#### Nonsense mutations

#### Missense mutations

<table>
<thead>
<tr>
<th>Individual</th>
<th>Exon</th>
<th>Mutation</th>
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</thead>
<tbody>
<tr>
<td>209-1 *</td>
<td>Exon 21</td>
<td>c.3823 G&gt;A</td>
</tr>
<tr>
<td>201-1</td>
<td>Exon 26</td>
<td>c.4340 C&gt;T</td>
</tr>
<tr>
<td>260-1</td>
<td>Exon 26</td>
<td>c.4348 T&gt;C</td>
</tr>
<tr>
<td>228-1</td>
<td>Exon 27</td>
<td>c.4409 A&gt;G</td>
</tr>
<tr>
<td>2644 *</td>
<td>Exon 30</td>
<td>c.4991 G&gt;A</td>
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</tbody>
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#### Deletions & Insertions

#### Splice site mutations

#### Rearrangements found by MLPA

### Mutations in the EP300 gene

<table>
<thead>
<tr>
<th>Individual</th>
<th>Exon</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>254-1</td>
<td>Exon 10</td>
<td>c.1942 C&gt;T</td>
</tr>
<tr>
<td>256-1</td>
<td>Exon 15</td>
<td>c.2877_2884 del 8</td>
</tr>
<tr>
<td>149-1</td>
<td>Exon 1</td>
<td>c.1200-?_94+? del</td>
</tr>
</tbody>
</table>

Table 1: List of all mutations found in the CBP gene, described in relation to GenBank file NM_004380, and EP300, GenBank file NM_001429.1, counting the A of the ATG start codon as nucleotide +1. The mutations are denoted according to the nomenclature as published by (den Dunnen and Antonarakis 2001). Del and dup means deletion and duplication; question marks indicate the breakpoints are unknown. The changes on DNA level have been confirmed by restriction digests or by second sequencing reactions. The changes on protein level listed here are predictions. All patients marked with an asterisk have mutations that have been published before in Kalkhoven et al. (2003) except for 127-2, which has been described as a deletion in mRNA by Petrij et al.(2000).
Large deletions and duplications at the CBP gene

Previous research suggests that approximately 10% of the mutations of RSTS patients are microdeletions affecting the CBP gene (Blough et al. 2000; Petrij et al. 2000). We performed Fluorescent in situ Hybridization (FISH) using five cosmids spanning the entire gene to detect such deletions when metaphase chromosome spreads of patients are available (Petrij et al., 2000). The recently developed technique of Multiple Ligation-dependent Probe Amplification (MLPA) can also be used to detect microdeletions on soluble genomic DNA (Schouten et al. 2002). Because that is the type of material available to us for the majority of our patients we set up MLPA on the CBP gene.

<table>
<thead>
<tr>
<th>E1278K</th>
<th>T1447I</th>
</tr>
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<tbody>
<tr>
<td>Hs: KKKNDTRLDEPFVDCKECG</td>
<td>Hs: HFFPRCLRQAVYHEILIG</td>
</tr>
<tr>
<td>Mm: KKKNDTRLDEPFVDCKECG</td>
<td>Mm: HFFPRLCLRQAVYHEILIG</td>
</tr>
<tr>
<td>Dm: EKKNDHLELEPVNCQECG</td>
<td>Dm: HFFPQYRTAVYHEILLG</td>
</tr>
<tr>
<td>209-1: KKKNDTRLDEPFVDCKECG</td>
<td>201-1: HFFPRLCLRQAVYHEILIG</td>
</tr>
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<tr>
<th>Y1450H</th>
<th>H1470R</th>
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<tr>
<td>Hs: RPRCLRTAVYHEILIGIFH</td>
<td>Hs: VKKLGYVTGHIWACPPSEG</td>
</tr>
<tr>
<td>Mm: RPRCLRTAVYHEILIGIFH</td>
<td>Mm: VKKLGYVTGHIWACPPSEG</td>
</tr>
<tr>
<td>Dm: RPRQYRTAVYHEILLGYMD</td>
<td>Dm: VKQLGYTMAHIWACPPSEG</td>
</tr>
<tr>
<td>260-1: RPRCLRTAVYHEILIGIFH</td>
<td>228-1: VKKLGYVTGHIWACPPSEG</td>
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<th>R1664H</th>
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<tbody>
<tr>
<td>Hs: LLSCDLMDGRTAPTLARD</td>
</tr>
<tr>
<td>Mm: LLSCDLMDGRTAPTLARD</td>
</tr>
<tr>
<td>Dm: LLSCDLMDGRTAPTLARD</td>
</tr>
<tr>
<td>2644: LLSCDLMDGRTAPTLARD</td>
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Figure 1: Conservation of amino acids predicted to change by missense mutations. All five mutations that are predicted to change the amino acid residue that we have found are situated in the highly conserved HAT domain. The changed residues are conserved in man (Homo sapiens), mouse (Mus musculus) and the fruit fly (Drosophila melanogaster).

The resolution of MLPA is related to the number of probes one uses. We made a set of 20 MLPA probe pairs covering most of the CBP gene. This allows us to screen for deletions that cannot be detected by FISH. Southern blotting could have been an alternative but is in our case impractical, if not impossible, because it requires too much DNA.
The quality of DNA is slightly more critical in MLPA than in a normal PCR, therefore, we could not screen all patients with MLPA that have been screened with DGGE and SSCP. In total we screened 53 patients and as controls we used material from 3 patients with known microdeletions already detected using FISH, including one with a deletion of the entire gene. Our MLPA analysis detected those positive controls flawlessly and we found a number of previously undetected mutations. In total we found 9 new deletions, ranging from single exon deletions to the entire gene. One deletion, of exon 2, has been described previously on RNA level (Petrij et al. 2000). At the time Southern blots did not reveal a deletion in the genomic DNA, therefore, it was not clear whether this was a genomic deletion or a splicing aberration. This mutation has been found in family 127, which consists of an affected mother and child, one of the very few cases of inherited RSTS.

Next to the nine deletions we have detected we also found a duplication in one individual. Patient 162-1 has a duplication of the first exon of the CBP gene. How this leads to the inactivation of this allele is not clear but a disease causing duplication of first exon has been described before in Opitz syndrome (Winter et al. 2003).

The exon 1 deletions and duplication were confirmed using extra probe pairs, one at the promoter region and three probe pairs in intron 1.

Mutations in the EP300 gene
Point mutation screening and MLPA analysis of CBP yielded a total of 36 mutations in 92 patients, suggesting that other genes could be involved in RSTS as well. The most likely candidate is the EP300 gene, coding for p300, on chromosome 22q13.1. That gene was screened as well, using the same approach. We used 37 DGGE fragments, covering approximately 79% of the coding sequence of EP300, with the remaining part was covered by 10 SSCP fragments. MLPA was performed with a set of 20 exon specific probe pairs.

Indeed, 3 inactivating mutations were detected in the EP300 gene (see fig.2). Two mutations were found using DGGE: one mutation, in exon 10, is a transition (c.1942 C>T) that converts the triplet coding for the arginine at position 648 into a stop codon. The other mutation, in exon 15, is a deletion of 8 nucleotides that predicts a frameshift from codon 959 with a stop codon after 7 amino acids. The exact location of the 8 bp deletion (c.2877_2884) was confirmed with an allele specific PCR. We analyzed DNA from the healthy parents of both patients with DGGE and sequencing and confirmed that the mutations occurred de novo. The biological parentage was confirmed by genotyping with 17 independent markers (data not shown). Both mutations lead to predicted proteins less than half their normal size, that do not contain the HAT domain. The third
mutation, a deletion of the first exon, was found using MLPA. Four probes revealed this deletion, two probes upstream of exon 1, one in exon 1 and the fourth in intron 1, close to the first exon. They all showed decreased signal whereas a probe in exon 2 showed a normal dosage (see fig.2c). It is probable that this deletion will lead to no expression from the affected allele.

Figure 2: Mutations in EP300 in RSTS patients. (a) Patient 254-1. DGGE of patient 254-1 and the healthy parents shows that only the affected child has the mutation. Subsequent sequence analysis revealed a transition c.1942 C>T that predicts p.Arg648X in this patient. (b) Patient 256-1. DGGE of family 256 with patient 256-1 shows a de novo mutation. The allele specific PCR confirms the exact location of the deletion seen by sequence analysis. The patient has an 8 bp deletion (c.2877_2884 del) in the following sequence: gcc tcctccatctactagta gCACAGAAGtg aat. The deleted region is indicated with capitals. The allele specific forward primer, consisting of the underscored nucleotides, skips the deleted part and anneals with 2 nucleotides after the deletion. Only the PCR on DNA from the patient shows a band of 168 bp, in the lanes with the PCR on DNA from the healthy parents only the prominently visible primer dimers can be seen. (c) Bar diagram of MLPA results for patient 149-1. MLPA reveals a deletion at the first exon of the EP300 gene. The bars indicate the dosage of the various probes used. The probes upstream of the first exon are at positions 787 to 716 and 54 to 5 bp before the transcription start site. In the figure they are indicated as –787 and –54 respectively. Ex refers to exon and Int to intron. The Y-axis represents the dosage of DNA: a dosage of 1 indicates the presence of the normal amount of DNA, that is, both alleles are present, whereas bars reaching approximately 0.5 typically indicate a deletion of one allele. In the figure it can be clearly seen that the deletion runs from the upstream region of exon 1 into intron 1 and that exons 2 and 3 are present for both alleles. The exact size of the deletion is unknown.
Discussion

We undertook a rigorous screening for point mutations, small deletions or insertions as well as larger deletions and duplications in the coding region of the CBP gene on genomic DNA of a large set of RSTS patients. There is no predominant type of mutation, nor is there a clear indication for clustering of mutations within the CBP gene. If we take a look, however, at missense mutations we see that they are all situated in the HAT domain of CBP. We have published some of these mutations previously and have shown that they affect the HAT activity of CBP. In addition, two papers each reported a de novo missense mutation that is within the HAT domain, clearly underpinning the importance of this domain in relation to the disorder (Murata et al. 2001; Bartsch et al. 2002). A study by Coupry et al. reported 4 putative missense mutations, of which only one was located in the HAT domain (Coupry et al. 2002). The sequence variations were not found in the other patients, and the affected residues were conserved in mouse.

We have found mutations in less than half of the patients, approximately 40%, which is comparable with the outcome of the study by Coupry et al. DGGE and SSCP analysis are, together with detection of nucleotide substitutions, only capable of identifying relatively small deletions and insertions. To detect larger deletions we chose to set up MLPA for the CBP gene and for EP300 as well. We have shown that MLPA is capable of detecting deletions in the CBP gene that were previously identified by FISH. Because we have probe pairs corresponding to the majority of exons in both CBP and EP300 our MLPA screening also negates the need for Southern blotting. The use of MLPA has increased the detection power for mutations, allowing us to find smaller deletions than could be detected with FISH.

The combined analysis of our samples with both MLPA and DGGE or SSCP nevertheless resulted in mutations being found in less than half of the patients. Although some of the patients we screened could possibly have a different syndrome that resembles RSTS we think the majority should be considered as true RSTS patients. Diagnosis of the syndrome has been performed by many clinicians but we do not see that some have a significantly better record in number of mutations found than others. Either the CBP gene is mutated at parts where we did not screen, such as the promoter or other regulatory elements, or the mutations are in other genes. The unscreened parts of the CBP gene may harbor some mutations but it is highly unlikely they will contain the majority of the missing 60%. Indeed, RSTS is genetically heterogeneous since we identified mutations in the EP300 gene.
A striking finding in our study is that the number of RSTS patients with \textit{EP300} mutations, now 3, is small compared to the number of RSTS patients, 36, with \textit{CBP} mutations. Possibly, this ratio of 1 to 12 represents the different chances of mutations occurring in these two genes. Alternatively, the \textit{EP300} gene could have an equal mutation rate as the \textit{CBP} gene but the carriers may not be diagnosed with RSTS. In view of this latter explanation it is interesting that we found many more polymorphisms in the \textit{EP300} gene including some that lead to amino acid changes (data not shown). Nevertheless, the majority of point mutations found in the \textit{CBP} gene are likely to lead to truncated proteins and two mutations in the \textit{EP300} gene are also predicted to truncate the protein so it is difficult to explain the skewed ratio with a different genotype/phenotype relationship. We therefore think that there is a different mutation rate between the two loci.

The \textit{CBP} gene contains an unstable region around exon 2. This region was designated as unstable because all translocation and inversion breakpoints in RSTS patients, except for one, could be found there, as well as all leukemia breakpoints where \textit{CBP} functions as a fusion partner. In addition, this same genomic piece of DNA proved very difficult to clone when the positional cloning of the RSTS syndrome gene took place (Giles et al. 1997). The deletion of exon 2 and the deletions and duplication of exon 1 may be caused by this unstable region. The instability in this region, however, cannot explain the majority of deletions found at the \textit{CBP} locus as most of these deletions have their breakpoints elsewhere (Petrij et al. 2000).

Although \textit{CBP} and \textit{p300} are probably redundant to a large extent, there are subtle but clear differences between the two proteins. During embryogenesis the two genes have similar but not completely overlapping expression patterns (Partanen et al. 1999). In addition, experiments with F9 teratocarcinoma cell lines showed that retinoic acid signaling is \textit{p300} dependent and does not require \textit{CBP}, whereas cAMP signaling depends on \textit{CBP} and not \textit{p300} (Kawasaki et al. 1998; Ugai et al. 1999). Recent work with transgenic mice indicated the importance of the acetyl transferase function of \textit{p300} in myogenesis, but the acetyl transferase function of \textit{Cbp} does not seem to be necessary for this process (Roth et al. 2003). The skeletal abnormalities found in heterozygous \textit{Cbp} knockout mice have not been reported for heterozygous \textit{Ep300} knockout mice (Tanaka et al. 1997). We, however, do not see clear phenotypical differences in RSTS patients with mutations in the \textit{EP300} gene instead of the \textit{CBP} gene. Patient 256-1 has a very short metatarsal bone (figure 3), not often seen in RSTS patients, however, similar dysmorphology has been found in a patient with a deleted \textit{CBP} gene (Petrij et al. 1995). Double heterozygous knockout mice for the \textit{Cbp} and \textit{Ep300} genes resemble the homozygous knockout mice for either gene, in that all three types of mice die \textit{in utero}, which led to the idea that the combined levels of...
CBP and p300 are critical during development (Yao et al. 1998). Our finding supports this hypothesis and reveals that even a relatively small decrease of either protein has significant developmental consequences. It is, however, unclear how a decrease of either protein leads to the specific features of RSTS. Perhaps the partial loss of p300 is compensated for by recruitment of CBP and subsequent depletion of CBP than leads to RSTS. Alternatively, both proteins could be involved in a common function and, therefore, the total dosage is required to prevent a syndrome like RSTS. If so, then this common function has a relationship with the HAT activity of the proteins because loss of only the HAT activity of CBP causes RSTS.

Interestingly, there is a direct link between HAT activity and long-term memory. Heterozygous Cbp knockout mice have diminished mental capabilities. Experiments on these knockout mice revealed that inhibiting histone deacetyltransferase could ameliorate the problems the mice have with their long-term memory (Alarcon et al. 2004). Transgenic mice with a dominant negative CBP gene, where only the HAT activity was ablated, also showed the long-term memory problems. Again, this could be reversed by a histone deacetylase inhibitor (Korzus et al. 2004). In view of these data it could be possible that other proteins with HAT activity, or with a function coupled to HAT activity, may also be involved in RSTS. After all, the three mutations we have found in the EP300 gene together with the CBP gene mutations still leaves us with more than half of the RSTS patients to be accounted for.

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References


