UNIVERSIDADE ESTADUAL DE CAMPINAS
Instituto de Biologia



Daniel Maragno Trindade

# Estudos estruturais e funcionais da proteína stanniocalcina-1 humana, um novo marcador de microambiente de leucemia



Tese de Doutorado a ser apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para a obtenção do Título de Doutor em Biologia Funcional e Molecular

Orientador: Prof. Dr. Jörg Kobarg
Co-orientador: Prof. Dr. José Andrés Yunes

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# Para refletir ...

"A verdadeira sabedoria chega a cada um de nós quando nos apercebemos quão pouco entendemos sobre a vida, nós mesmos, e o mundo à nossa volta"

(Sócrates)

"Se ouvires uma voz dentro de você dizendo: 'Você não é um pintor' Então, por todos os meios pinte ... e aquela voz será silenciada."

(Vincent Van Gogh)

"No! Try not. Do. Or do not. There is no try."

"Mind what you have learned. Save you it can."

(Yoda – Star Wars)

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# Lista de abreviaturas e siglas

| | |
|---|---|
| **3AT** | 3-aminotriazol |
| **Å** | angstron ou $10^{-10}$ metros (*Ångström*) |
| **AD** | domínio de ativação transcricional da proteína Gal4 (*Activation domain*) |
| **ATF** | fator ativador da transcrição (*activating transcription factor*) |
| **ATP** | trifosfato de adenosina (*Adenosine triphosphate*) |
| **BCFH** | biblioteca de Cérebro Fetal Humano (*Human fetal Brain Library*) |
| **BD** | domínio de ligação ao DNA da proteína Gal4 (*Binding domain*) |
| **BM** | medula óssea (*Bone Marrow*) |
| **BMEC** | células endoteliais da medula óssea (*Bone Marrow Endothelial Cells*) |
| **BSA** | albumina sérica bovina (*Bovine Serum Albumin*) |
| **$Ca^{2+}$** | íon divalente de cálcio |
| **CD** | Dicroísmo Circular (*Circular Dichroism*) |
| **cDNA** | DNA complementar |
| **CDS** | Seqüencia Codificadora (*Coding Sequence*) |
| **CHO** | linhagem de células de ovário de hamster chinês (*Chinese hamster ovary* cell line) |
| **$Co^{2+}$** | íon divalente de cobalto |
| **CS** | Corpúsculo de Stannius |
| **$Cu^{2+}$** | íon divalente de cobre |
| **Da** | Dalton (unidade de medida de massa utilizada para expressar a massa de partículas atômicas) |
| **deg** | graus (*degrees*) |
| **DKK1** | Dickkopf-1 |
| **DLS** | espalhamento dinâmico de luz (*Dynamic Light Scattering*) |
| **DMEM** | meio *Eagle* modificado por Dulbeco (*Dulbeco's Modified Eagle Medium*) |
| **dmol** | decimol ou $10^{-1}$ mol |
| **DNA** | ácido desoxiribonucleico (deoxiribonucleic acid) |
| **DOTAP** | N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl-sulfate |
| **DTT** | 1,4 ditiotreitol |
| **EDTA** | ácido etilenodiaminotetraacético |
| **EGFP** | versão "intensificada" da **GFP** (*Enhanced GFP*) |
| **ER** | retículo endoplasmático |
| **ERK** | proteína quinase regulada por sinal extracelular (*extracellular signal-regulated kinase*) |
| **ERN1** | **ER** para o núcleo 1 (*Endoplasmic reticulum to nucleous 1 or IRE1*) |
| **ESI/Q-TOF** | ionização por eletrospray (*Electrospray Ionization*) acoplada a um analisador de massas quadrupolo-tempo de voo (*Quadrupole Time-Of-Flight*) |

**EST** marcador de sequência espressa (*expressed sequence tag*)

**FCS** soro fetal bovino (*Foetal Calf Serum*)

**FGF** fator de crescimento de fibroblastos (*Fibroblast growth factor*)

**FPLC** cromatografia líquida de ata pressão (*Fast Performance Liquid Chromatography*)

**GAPDH** gliceraldeido-3-fosfato desidrogenase (*Glyceraldehyde-3-phosphate dehydrogenase*)

**GFP** proteína fluorescente verde (*Green Fluorecent Protein*)

**GSH** glutationa reduzida

**GSK3** quinase 3 da glicogenio sintase (*Glycogen synthase kinase 3*)

**GSSG** glutationa oxidada

**GST** glutationa-S-transferase

**HBSS** solução salina balanceada de *Hank* (*Hank's Balanced Salt Solution*)

**HDACs** desacetilases de histonas (*Histone deacetilases*)

**HGF** fator de crescimento de hepatócitos (*Hepatocyte growth factor*)

**HIF-1** fator 1 induzível por hipóxia (*Hypoxia inducible factor 1*)

**HighFive™** linhagem de células derivadas do clone BTI-TN-5B1-4 da larva de *Trichopulsia ni*

***HIS3*** gene que codifica a imidazol glicerol-fosfato desidratase, a qual cataliza o sexto passo na biossíntese de histidina em leveduras *Saccharomyces cerevisiae*

**HRP** peroxidase de rabanete (*Horseradish Peroxidase*)

**IEX** cromatografia de troca iônica (*Ion Exchange cromatography*)

**IL** interleucina

**IPTG** isopropil beta-D-galactosídeo;

**IRE1** **ERN**

**kDa** quilo Dalton

**Kg** quilograma ou $10^3$ gramas

***LacZ*** gene que codifica para a enzima β-galactosidase

**LB** (meio) Luria Bertani

**LMNA** Laminina A/C

**m.o.i.** multiplicidade de infecção (*multiplicity of infection*)

**MAP** proteína ativada por mitógenos (*Mitogen-activated protein*)

**MAPK** proteína quinase ativada por mitógenos (*Mitogen-activated protein kinase*)

**MCS** sítio de clonagem múltipla *(Multiple cloning site*)

**MEK** proteína kinase de **ERK** (*ERK kinase or MAPKK*)

**MES** ácido 4-morfolino etano sulfônico (*4-Morpholineethanesulfonic acid*)

**mg** miligrama ou $10^{-3}$ grama

**mRNA** RNA mensageiro

**MS** espectrometria de massa (*Mass Spectrometry*)

**NADH** nicotinamida adenina dinucleotídeo (*Nicotinamide adenine dinucleotide*)

**NBs** corpusculos nucleares (*Nuclear Bodies*)

**NFκB** fator nuclear kappa B (Nuclear factor k B)

**ng** nanograma ou $10^{-6}$ grama

**$Ni^{2+}$** íon divalente de níquel

**Ni-NTA** ácido níquel-nitrilotriacético

**nm** nanômetro

**NMWL** limite nominal de peso molecular (*Nominal Molecular Weight Limit*)

**NRMSD** raiz quadrada normalizada do desvio padrão médio (*Normalized Root Mean Square Deviation*)

**NSD** discrepância espacial normalizada (*Normalized Spatial Discrepancy*)

**PCR** reação em cadeia da polimerase (*Polimerase Chain Reaction)*

**PERK** proteína quinase do retículo endoplasmático semelhante à **PRK** (*PKR-like Endoplasmic Reticulum Kinase*)

**PKA** proteína quinase A (*Protein Kinase A*)

**PKC** proteína quinase C (*Protein Kinase C*)

**PKG** proteína quinase G (*Protein Kinase G*)

**PMA** 12-miristato 13-acetato de forbol (*Phorbol 12-myristate 13 acetate*)

**Polylinker** sítio de clonagem múltipla ou MCS (*Multiple cloning site*)

**PRK** Fosfo-ribulo-quinase (*Phosphoribulokinase*)

**PRL-3** proteína tirosino-fosfatase de figado em regeneração 3 (*Protein-tyrosine phosphatase of regenerating liver 3*)

**PVDF** fluoreto de polivilidina (*Polyvinylidene fluoride*)

**qPCR** **PCR** em tempo real quantitativo (*Real-time Quantitative PCR*)

**RNA** ácido ribonucléico (ribonucleic acid)

**RSK** quinase ribossomal de 90 kDa S6 (*90-kDa Ribosomal S6 Kinase [$pp90^{RSK}$]*)

**SAXS** espalhamento de raios-X a baixo ângulo (*Small Angle X-ray Scattering*)

**SERCA** bomba $Ca^{2+}$ ATPase do retículo endo/sarcoplasmático (*Sarco/Endoplasmic reticulum calcium pump*)

**Sf9** linhagem de células derivadas da larva de *Spodoptera frugiperda*

**SIM** motivo de interação com **SUMO** (*SUMO Interacting Motif*)

**STC1** stanniocalcina-1

**STC2** stanniocalcina-2

**SUMO1** proteína modificadora relacionada a ubiquitina 1 (*Small ubiquitin-related modifier 1*)

**TBS** solução salina tamponada com Tris (*Tris Buffered Saline*)

**TCEP** tris(2-carboxyethil)fosfina

**TKR** receptor tirosino-quinase (*Tyrosine Kinase Receptors*)

**TNF** Fator de necrose tumoral (*Tumor necrosis factor*)

**Tris** tris(hidroximetil)aminometano

**TTBS** **TBS** contendo o detergente Tween-20

**UPR** resposta à proteínas desenoveladas (*Unfolded protein response*)

**UV** ultra violeta

**VEGF** Fator de crescimento vaso-endotelial (*Vascular endothelial growth factor*)

**$Zn^{2+}$** íon divalente de zinco

**µg** micrograma

# I. Resumo

Staniocalcinas (STCs) representam uma pequena família de hormônios glicoprotéicos, encontrados em todos os vertebrados e composta por STC1 e STC2, que foram inicialmente implicados na homeostase de cálcio e recentemente também foram implicados em vários outros processos. Stanniocalcina-1 (STC1), o primeiro membro encontrado, foi originalmente descoberto em peixes ósseos e posteriormente identificado em humanos onde parece ter um papel, embora ainda obscuro, na carcinogênese e angiogênese. Nos seres humanos STC1 pode ser encontrado em duas formas: um dímero ou um grupo de variantes de alto peso molecular coletivamente chamados bigSTC. Uma vez que ambas as células leucêmicas e os tumores sólidos dependem vascularização, a angiogênese é um passo fundamental na interação tumor-hospedeiro e essencial para a progressão do câncer. Análises prévias de microarray resultaram na identificação de vários genes ativados em células endoteliais da medula óssea (BMEC) modulados pela presença de células leucêmicas. Apresentamos aqui STC1 como um marcador microambiente de medula óssea (BMM) durante leucemia linfoblástica aguda (LLA) pela validação dos dados prévios de microarray através de PCR em tempo real quantitativos. Em vista da falta de informações funcionais e estruturais sobre STC1 realizamos: (1) um screen no sistema de duplo híbrido em levedura, a fim de encontrar algumas das proteínas que interagem com a STC1 humana, e (2) uma caracterização estrutural inicial à baixa resolução desta proteína. Fomos capazes de construir um mapa interação proteína-proteína, obtido a partir dos 22 interactantes encontrados em screen de duplo híbrido em levedura. As proteínas encontradas apresentam-se em vários compartimentos celulares nos quais STC1 já foi demonstrada para estar presente, e essas novas informações podem ajudar a esclarecer como e qual o papel STC1 desempenha nesses locais. A fim de fornecer informação estrutural sobre STC1, realizamos análises bioquímicas e estruturais da proteína recombinante STC1 com 6xHis tag produzida em células de inseto utilizando o sistema baculovírus. A análise de dicroísmo circular confirmou a predição in silico do elevado conteúdo de alfa-helices. A análise por espectrometria de massas forneceu os dados experimentais que confirmaram o padrão conservado de pontes dissulfeto anteriormente descrito para STC1 de peixes. Finalmente, os dados de espalhamento de raios-X a baixo ângulo demonstraram que, STC1 adota uma estrutura dimérica, ligeiramente alongada em solução fornecendo deste modo os primeiros dados estruturais à baixa resolução desta família de proteínas. Além disso, foi possível obter proteína suficiente e de elevado grau de pureza para realizar ensaios cristalização que resultaram em cristais os quais difrataram a boa resolução.

# II. Abstract

Staniocalcins (STCs) represent a small family of glycoprotein hormones found in all vertebrates composed by STC1 and STC2, which have been initially implicated in calcium homeostasis and also recently implicated in several other processes. Stanniocalcin-1 (STC1), the first member found, was originally discovered in bony fishes and later identified in humans were it seems to have a role, although still unclear, in carcinogenesis and angiogenesis. In humans STC1 can be found in two forms: a dimer or a group of higher molecular weigh variants collectively called bigSTC. Since both leukemic cells and solid tumors depend on vascularization, angiogenesis is a fundamental step in tumor-host interaction and essential to the cancer progression. Previous microarray analysis resulted in the identification of several activated genes in bone marrow (BM) endothelial cells (BMEC) modulated by presence of leukemic cells. Here we present STC1 as a BM microenvironment marker during acute lymphoblastic leukemia (ALL) by validating previous microarray data by quantitative RealTime-PCR. In view of the lack of functional and structural information on STC1 we performed (1) a yeast two hybrid screen in order to find some of the human STC1 interacting proteins and (2) a initial structural low-resolution characterization of this protein. We were able to construct a protein-protein interaction map, derived from the 22 interactants found in our yeast two-hybrid screen. The proteins found are located in several cellular compartments in which STC1 has already been shown to be present, and the new information might help to clarify how and which role it performs at these sites. As a means to provide structural information about STC1, we performed biochemical and structural analyses of recombinant STC1 6xHis tagged protein produced in insect cells by using the baculovirus system. Circular dichroism analysis confirmed the *in silico* predicted high alpha-helical content. By mass spectroscopy analysis we provided experimental data that confirmed the conserved disulfide pattern previously described for fish STC1. Finally Small Angle X-ray Scattering data demonstrated that STC1 adopts a dimeric, slightly elongated structure in solution, providing there by the first low resolution structural data of this family of proteins. Additionally, we could obtain enough protein of high purity to perform crystallization trials that resulted in crystals diffracting at good resolution.

# III. Introdução

## A. CÉLULAS, GENES E CÂNCER

O crescimento e a diferenciação celular são processos altamente regulados, que são direcionados por um programa genético e modificados por estímulos ambientais. O número de células em um organismo adulto é mais ou menos constante, mantendo-se estável devido a processos de divisão e morte celular (homeostase). A vida média dos diferentes tipos celulares do corpo humano varia desde poucos dias, no caso de glóbulos brancos, até vários anos, no caso das células do sistema nervoso central (Spalding *et al.*, 2005).

Defeitos adquiridos no material genético que afetam a expressão ou o funcionamento de genes envolvidos na regulação do crescimento e diferenciação celular causam o câncer. As células cancerosas, quando comparadas às células normais, apresentam três diferenças: não são limitadas no seu crescimento e divisão celular, não se diferenciam normalmente e não morrem de acordo com o programa normal de morte celular. Certas células que acumulam mutações podem ganhar vantagens de crescimento e podem finalmente escapar do controle da homeostase celular de um organismo saudável. Três grupos de genes são afetados por mutações e contribuem para o processo de carcinogênese: oncogenes, genes supressores de tumor e genes envolvidos no processo de programa de morte celular conhecido como apoptose (Vogelstein & Kinzler, 1993).

Proto-oncogenes sofrem freqüentemente mutações pontuais ou têm sua expressão alterada, tornando-se versões mutadas de genes normais envolvidos no processo de crescimento celular, ou também chamados oncogenes. Tais modificações podem promover um ganho de função para o produto gênico, que por sua vez, proporciona vantagem de crescimento celular (Bishop, 1991). Por outro lado, mutações nos genes supressores de tumor ou genes envolvidos no processo de apoptose, podem causar a perda de suas funções, e deste modo também contribuir para o progresso do desenvolvimento tumoral. Os genes envolvidos na morte celular podem ser divididos em dois grupos: os pró-apoptóticos e os anti-apoptóticos. Super-expressão de proteínas anti-apoptóticas, assim como, redução da atividade ou supressão das pró-apoptóticas, podem tornar as células resistentes à apoptose e, conseqüentemente, resistentes a um grande número de drogas que induziriam a apoptose em células normais (Martin & Green, 1995).

## B. MARCADORES MOLECULARES

Cânceres são classificados pelo tipo de célula que se assemelha ao tumor e, por conseguinte, o tecido que se presume ter sido a origem do tumor. Alterações gênicas podem

resultar em diferenças entre o mesmo tipo de câncer gerando perfis distintos de agressividade, proliferação e invasividade em função do tipo de proteína afetada ou da severidade funcional da alteração ou dos mecanismos que esta proteína afetada controla ou regula.

Marcadores moleculares de câncer são genes ou proteínas que apresentam expressão e ou função alterada em células cancerosas, em comparação com células normais (Sidransky, 2002). Estes marcadores podem ser detectados, por exemplo, por biopsia do tecido tumoral seguida da análise deste tecido com anticorpos específicos ou métodos moleculares baseados em oligonucleotídeos de DNA; como também podem ser detectadas em fluidos corpóreos tais como: sangue, urina e líquido cerebro-espinal. A identificação e detecção de novos marcadores moleculares são de suma importância para o diagnóstico, acompanhamento da resposta à terapia, predição do prognóstico, além de possibilitar o desenvolvimento de novas drogas antitumorais. Além disso, a seleção de marcadores específicos para um dado tipo de câncer pode permitir a classificação de pacientes em diferentes grupos de risco de acordo com o grau de progressão do câncer, permitindo a adequação, já antes do início do tratamento, do nível de agressividade do regime terapêutico a ser adotado (Caldas & Ponder, 1997; Golub *et al.*, 1999; Vickers *et al.*, 2008). A identificação de novos marcadores tumorais é feita, principalmente, com o uso de técnicas que permitem um alto fluxo (*high through put*) de amostras, tais como micro-arranjos de DNA ("DNA microarray") e espectroscopia de massas de proteínas ou "proteômica" (Verma *et al.*, 2001). A técnica de micro-arranjos de DNA permite a análise simultânea dos níveis de expressão de milhares de genes expressos (transcriptoma), enquanto que a espectrometria de massa (MS) analisa a população protéica (proteoma) da amostra em estudo, qualitativamente e quantitativamente.

## C. ESTUDOS DE ESTRUTURA E FUNÇÃO DOS MARCADORES TUMORAIS

A elucidação da estrutura tridimensional dos componentes protéicos envolvidos nos processos carcinogênicos (marcadores tumorais) tem papel chave para o entendimento dos seus mecanismos moleculares e sua regulação, servindo como base para o desenho racional de drogas e outras moléculas antitumorais.

Vários exemplos bem sucedidos já estão documentados na literatura, como por exemplo, o que envolve os receptores de tirosina-quinases (TKR). Os TKRs são proteínas com papel crucial na transdução de sinais celulares e frequentemente apresentam expressão descontrolada, ou mesmo hiperativada, em células cancerosas, contribuindo para o crescimento desregulado do tumor. Exemplos de sucesso no desenvolvimento de moléculas antitumorais envolvendo TKRs são: (i) o trastuzumab, um anticorpo monoclonal que interfere com os receptores HER2/neu (conhecido como *Herceptin*), (ii) o imatinib, um derivado de 2-

phenylaminopyrimidine que é um potente inibidor específico das TKRs (comercializado pela Novartis sob o nome de *Gleevec*) e (iii) o gefitinib, outro inibidor seletivo de receptores HER1/ErbB-1 (comercializado pela Teva com o nome de *Iressa*) (Ghosh *et al.*, 2001; Bennasroune *et al.*, 2004).

Outro exemplo é a fosfatase PRL-3, descoberta durante uma busca por marcadores moleculares associados à progressão de câncer coloretal do estágio primário ao metastático, cuja expressão é mais elevada neste ultimo estágio (Saha *et al.*, 2001). A resolução da estrutura da proteína PRL-3 possibilitou a identificação de características estruturais únicas não encontradas nas demais fosfatases (Kim *et al.*, 2004; Kozlov *et al.*, 2004), bem como permitiu o desenho de inibidores desta (Park *et al.*, 2008).

O desenho de inibidores de kinases tem se beneficiado imensamente de dados cristalográficos para a elucidação de modos de ligação e de rearranjos conformacionais inesperados induzidos por inibidores. De fato, a maioria das descobertas de inibidores de quinases acontece muito mais em conseqüência do desenho racional de drogas do que via screenings em larga escala e otimização empírica com base nos ensaios de atividades/funcionais (Zhang *et al.*, 2009).

## D. LEUCEMIA

Leucemia é um termo que abrange um amplo espectro de doenças, que por sua vez faz parte do grupo de doenças ainda maior chamado neoplasias hematológicas. A palavra leucemia tem origem grega (leukos /λευκός/, "branco"; aima /αἰμα/, "sangue") e é usada para definir o câncer do sangue ou medula óssea, caracterizado por uma proliferação anormal de células sanguíneas, geralmente leucócitos (células brancas do sangue). A Leucemia é classificada de acordo com dois aspectos clinico-patologicos. O primeiro aspecto leva em conta o grau de diferenciação da célula que originou o câncer podendo ser dividida em aguda e crônica (Figura 1). A Leucemia aguda é caracterizada pelo rápido aumento de células sanguíneas imaturas e essa forma de leucemia é a forma mais comum de leucemia em crianças. Já a leucemia crônica é distinguida pelo excessivo acumulo de células sanguíneas relativamente maduras, mas ainda anormais, levando tipicamente levando meses ou anos para progredirem; todavia estas células são produzidas a uma velocidade muito mais elevada que as células normais, resultando em um número elevado de glóbulos brancos no sangue. Deste modo este subtipo de leucemia acomete principalmente pessoas mais velhas porém pode acometer qualquer faixa etária. O segundo aspecto leva em conta qual a linhagem sanguínea afetada, linfóide e mielóide. Combinando esses dois aspectos obtem-se quatro principais categorias: Leucemia linfóide (ou linfoblástica ou linfocítica) aguda (LLA), Leucemia linfóide crônica (LLC), Leucemia mielóide (ou mielogênica)

aguda (LMA) e leucemia mielóide crônica (LMC) (Misaghian *et al.*, 2009). Destas, a LLA é o tipo mais comum de leucemia em crianças jovens, podendo afetar também adultos, especialmente aqueles maiores de 65 anos. O tratamento padrão envolve quimioterapia e radioterapia. Os subtipos de LLA podem incluir LLA tipo B, LLA tipo T, leucemia de Burkitt, e leucemia aguda bifenotípica.

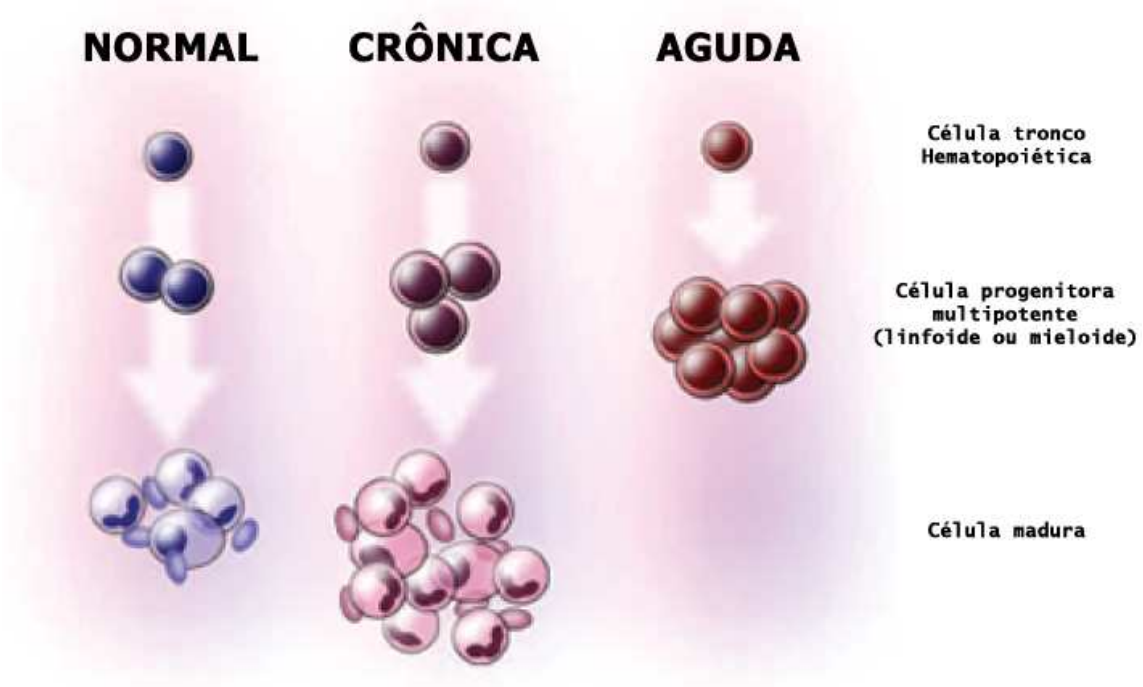


**Figura 1. Desenvolvimento das células normais em comparação com os tipos de leucemia crônica e aguda.** Modificado a partir de http://stemcells.nih.gov/info/2006report .

Segundo dados da Organização mundial da saúde, a cada ano aproximadamente 250.000 crianças e adultos são diagnosticados com um tipo de leucemia, o que representa cerca de 2,5% de todas as pessoas diagnosticadas com câncer. Todavia, o progresso no diagnóstico e tratamento da leucemia infantil tem transformado essa doença, antes fatal, em um grupo de malignidades que agora são curadas em muitas crianças. Esse progresso ocorreu devido ao esforço conjunto de investigações científicas e o trabalho de grupos de triagem clinica em cooperação nacional e internacional. Os avanços nas técnicas de citogenética, imunofenotípicas e análises de genética molecular permitiram a identificação de marcadores clínicos em células leucêmicas. Esses novos conhecimento têm contribuído para o desenvolvimento de protocolos de tratamento designados para sub-grupos específicos de pacientes com leucemia infantil (Colby-Graham & Chordas, 2003).

## E. INTERAÇÃO LEUCEMIA, MICROAMBIENTE TUMORAL E ANGIOGÊNESE

O desencadeamento da angiogênese é um processo fundamental da interação tumor-hospedeiro e essencial para a progressão do câncer (Folkman, 1995). As leucemias, bem como os tumores sólidos, são dependentes de vascularização e apresentam um aumento significativo de angiogênese estimada pela densidade da microvasculatura (DMV), demonstrando o papel importante da angiogênese na medula óssea durante a patogênese e progressão de neoplasias hematológicas (Hussong *et al.*, 2000; Negaard *et al.*, 2009). Apesar do fato de que as leucemias não apresentam uma associação direta entre a DMV e o prognóstico dos pacientes (Pule et al., 2002), inibidores de angiogenese mostraram-se eficazes como agentes anti-tumorais em modelos animais, indicando a dependência da angiogenese nas leucemias (Iversen *et al.*, 2002; Schuch *et al.*, 2005).

As células endoteliais da medula óssea (BMEC, *bone marrow endothelial cells*) formam microcapilares e vasos sangüíneos, e têm um papel importante no desenvolvimento do estroma da medula óssea como também na sustentação, estimulação e sobrevivências das células da leucemia. De fato a LLA está associada a um aumento significativo da angiogênese na medula óssea e dos níveis séricos de fatores de angiogênese (VEGF, TNF-alfa entre outros). Por outro lado, as BMEC promovem a sobrevivência das células da LLA e a interação entre esses dois típos de células confere vantagens proliferativas e adaptativas às células leucêmicas.

O grupo do Prof. Dr. José Andrés Yunes (Centro Boldrini) vem estudando esse tipo de interação a fim de entender o papel do microambiente medular, e principalmente do endotélio, para a progressão das células tumorais leucêmicas. Este grupo levanta a hipótese que o "grau de ativação" das células endoteliais seja importante para o progresso/agressividade da leucemia. Eles realizaram estudos de micro arranjos de DNA (Yunes, comunicação pessoal) e identificaram um grande conjunto de genes que são super expressos em células BMEC após sua interação/incubação, ou com células leucêmicas, ou com plasma de crianças com leucemia. Dentre os genes encontrados por Yunes estão Dickkopf-1 (DKK1), Stanniocalcina-1 (STC1) e a proteína do Mammalian Gene Colection (Strausberg *et al.*, 1999) CGI-109; cujas quantidades de mRNA aumentaram 3, 4 e 8 vezes, respectivamente, nas células tratadas. Esses dados indicam que estas proteínas são super expressas no tecido endotelial do microambiente medula óssea tomada pela leucemia e que estas proteínas têm potencial de marcadores moleculares da angiogênese em curso. A seguir daremos ênfase às família das Stanniocalcinas, visto que a proteína Stanniocalcina 1 foi por nos selecionada e validada como marcador de microambiente de leucemia (vide Artigo I na seção resultados).

## F. A FAMÍLIA DE PROTEÍNAS DAS STANNIOCALCINAS

As Stanniocalcinas (STC) representam uma pequena família de hormônios glicoprotéicos secretados, composta pelas proteínas STC1 e STC2 as quais compartilham seqüências de aminoácidos altamente conservadas desde vertebrados aquáticos até os terrestres (Wendelaar Bonga *et al.*, 1989; Pandey, 1994; Wagner *et al.*, 1995; Tanega *et al.*, 2004; Hang & Balment, 2005; Shin *et al.*, 2006; Wagner & DiMattia, 2006).

O gene STC1 de mamíferos foi originalmente identificado e clonado durante uma pesquisa de genes relacionados com câncer (Chang *et al.*, 1995). Acreditava-se inicialmente que a STC1 de mamíferos apresentaria uma conservação da função apresentada na STC1 de peixes, atuando na homeostase mineral como um hormônio anti-hipercalcêmico (Lafeber *et al.*, 1988; Olsen *et al.*, 1996; Wagner *et al.*, 1997; Madsen *et al.*, 1998). Além disso Zhang e colaboradores (1998a) mostraram que tanto a proteína STC1 humana produzida em baculovírus como em células de hamster (CHO) produzem o mesmo efeito inibitório do transporte de cálcio em brânquias de peixes que a proteína obtida de peixes, mostrando a alta conservação da sua função. Contudo, fica cada vez mais claro que as STCs tenham expandido seus papéis em mamíferos, sendo esta suposição baseada no fato de que as STCs, em humanos, além de possuírem um padrão de expressão abrangente em diversos tecidos normais adultos (Chang *et al.*, 1995; Varghese *et al.*, 1998; Worthington *et al.*, 1999; Paciga *et al.*, 2002; Serlachius *et al.*, 2002; Yoshiko & Aubin, 2004; Wagner & DiMattia, 2006), também apresentam uma expressão diferencial durante a embriogênese (Zhang *et al.*, 1998b; Franzen *et al.*, 2000; Jiang *et al.*, 2000; Yoshiko *et al.*, 2003; Serlachius *et al.*, 2004; Serlachius & Andersson, 2004). Existem cada vez mais evidências de um papel da STCs no câncer, tendo ambas proteínas sido relacionadas com carcinomas de mama (Bouras *et al.*, 2002; Welcsh *et al.*, 2002; Wascher *et al.*, 2003; Yamamura *et al.*, 2004; Joensuu *et al.*, 2008; Raulic *et al.*, 2008), colon (Fujiwara *et al.*, 2000; Gerritsen *et al.*, 2002; Yeung *et al.*, 2005; Law *et al.*, 2008b; Law *et al.*, 2008a), naso-faringeo (Yeung *et al.*, 2005), ovariano (Ismail *et al.*, 2000; Yeung *et al.*, 2005) e leucêmico (Tohmiya *et al.*, 2004), dentre outros (para revisão Fujiwara *et al.*, 2000; Chang *et al.*, 2003; Wascher *et al.*, 2003).

O genoma humano e o de rato codificam proteínas STC1 de 247 aminoácidos (Chang *et al.*, 1996; Chang *et al.*, 2003). Os primeiros 204 aminoácidos apresentam 92% de similaridade com a seqüência da STC1 de salmão, incluindo o sitio de N-glicosilação do tipo Asn-x-Thr/Ser (NxT/S) (Hulova & Kawauchi, 1999; Chang *et al.*, 2003). Entretanto existe pouca conservação nos últimos 43 resíduos do C-terminal comparando-se a STC1 de peixe com as STC1 e STC2 humanas (Figura 2), sugerindo que a principal atividade biológica das STCs deva ser mediada através da sua porção N-terminal (Ishibashi & Imai, 2002; Gerritsen & Wagner, 2005).

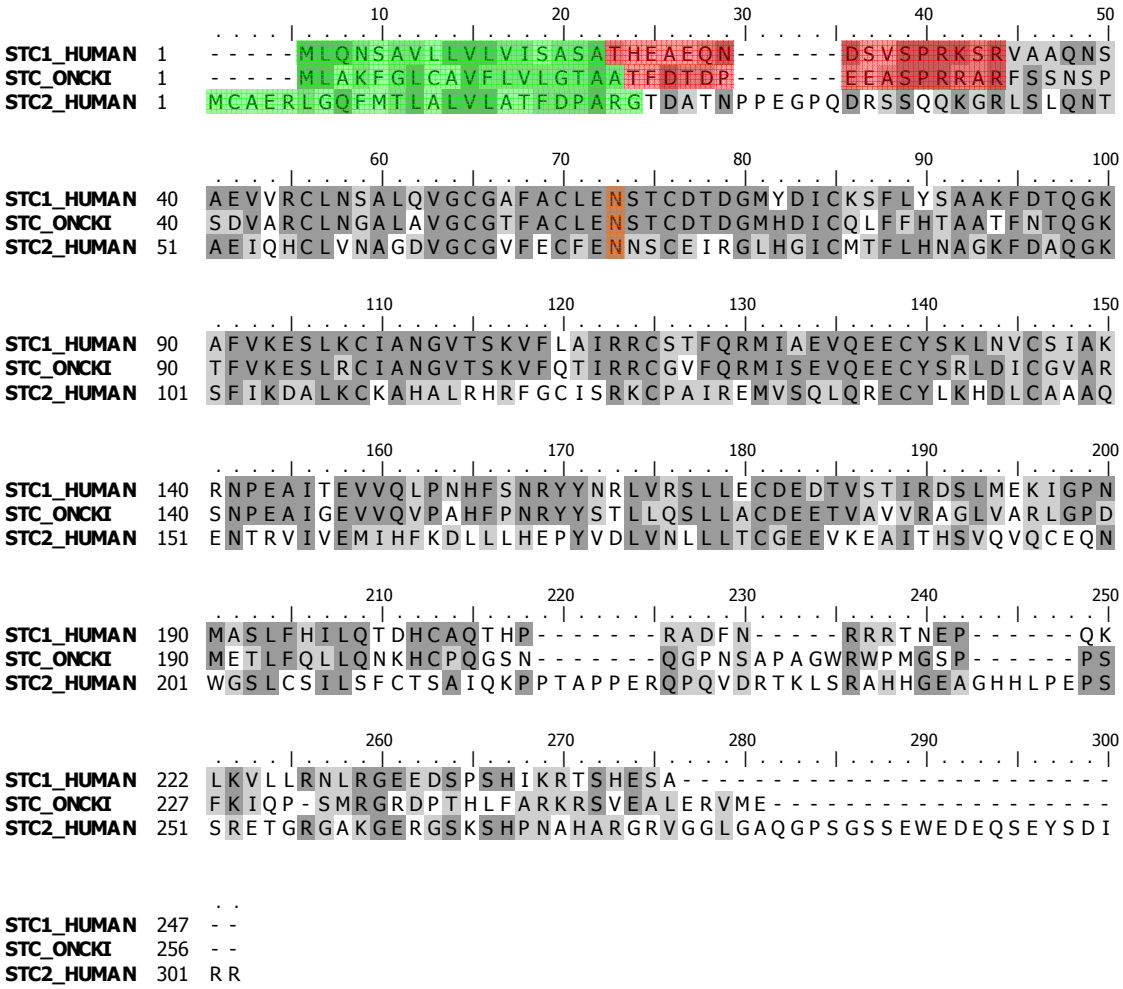


**Figura 2. Alinhamento entre sequencias de STC 1 e 2 humanas e a STC de truta arco-íris (*Oncorhynchus mykiss*, família Salmonidae).** Marcações em **tons de cinza** indicam conservação (de acordo com a matriz de substituição PAM250), em **verde**: peptídeo sinal, **vermelho**: pró-peptídeo e **laranja**: sitio de N-glicosilação.

Já o cDNA da STC2 codifica uma proteína de 302 aminoácidos (55 aminoácidos mais longa do que a STC1 humana) que possui 34% de identidade com STC1 humana e de enguias, sendo os resíduos de 24 a 101 (N-terminal) a porção mais idêntica (50% de identidade e 73% de similaridade com a STC1 humana). A porção da proteína STC2 após a posição 101 apresenta, no entanto, menor identidade (23%) para com a STC-1 humana (Moore *et al.*, 1999). Uma característica conspícua da proteína STC2 é a presença de 15 resíduos de histidina, ou seja, cinco vezes o número observado na STC1 de enguia e mais do dobro do número encontrado na STC1 humana. Quatro destes resíduos de histidina apresentam-se agregados em dois pares na região C-terminal da proteína e podem interagir com íons metálicos bivalentes, como $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$e $Cu^{2+}$ (Ishibashi & Imai, 2002), tendo este cluster de histidinas sido utilizado para purificar STC-2 em uma coluna de $Ni^{2+}$ (Moore et al, 1999). Considerando-se o alto grau de conservação

da porção N-terminal e no padrão de resíduos cisteína conservados (Gagliardi *et al.*, 2005) torna-se tentador assumir que STC1 e STC2 apresentem, pelo menos em parte, sobreposição de funções.

Em mamíferos parece existirem duas formas diferentes de STC1: (1) a forma convencional, também conhecida como STC50, que se apresenta como um dímero de 56 kDa constituído por monômeros de ~28 kDa, e (2) um número de variantes de maior peso molecular de STC coletivamente referidos como "bigSTC" (Paciga *et al.*, 2002; Paciga *et al.*, 2003; Paciga *et al.*, 2004; Paciga *et al.*, 2005a; Paciga *et al.*, 2005b; Hasilo *et al.*, 2005). Pelo menos três pesos moleculares de bigSTC foram descritos (84, 112 e 135 kDa) e têm sido relatados expressos em adipócitos, células adrenocorticais (Paciga *et al.*, 2005a; Paciga *et al.*, 2005b) e ovários (Paciga *et al.*, 2002; Paciga *et al.*, 2003; Paciga *et al.*, 2004). A fim de explicar o 'ganho de massa' nas bigSTC tem se sugerido que estas possuam algum tipo de modificações pós-traducionais como glicosilação (Paciga *et al.*, 2002; Chang *et al.*, 2003) ou fosforilação (Jellinek *et al.*, 2000), bem como a existência de exons adicionais ainda não caracterizados (Paciga *et al.*, 2005a). Um fato em cocordancia com a hipótese dos exons alternativos é que a forma monomérica da bigSTC1 apresenta cerca de ~10 kDa a mais que o monômero de STC50. Outra possibilidade é a formação de tri-(84 kDa), tetra-(112 kDa), ou mesmo pentameros (140 kDa), embora neste caso estejamos levando em conta somente os valores obtidos para o monômero de STC50 (~28 kDa), e não o tamanho observado do monômero de bigSTC (~38 kDa). Cabe ainda destacar que a variante de bigSTC de 135-kDa encontrada em células adrenocorticais é resistente a redução química, tal qual a STC50 derivada da matriz mitocondrial (Paciga *et al.*, 2005a), sugerindo talvez uma estruturação quaternária da proteína que a torne menos susceptível à redução química. Ademais em peixes mais primitivos o último resíduo de cisteína no C-terminal de STC1, supostamente envolvido na sua dimerização, não é conservado sendo substituído por resíduos de arginina ou histidina, dando assim origem a uma forma estritamente monomérica dessa proteína (Amemiya *et al.*, 2002; Amemiya *et al.*, 2006). Finalmente, embora as formas diméricas de STC1 tenham sido descritas (Wagner *et al.*, 1992; Yamashita *et al.*, 1995; Hulova & Kawauchi, 1999), a questão de sua multimerização e a ocorrência de diversas formas de peso molecular mais elevado em certas circunstâncias ainda permanecer para ser esclarecida.

# IV. Objetivos

## A. Objetivo Geral

Este estudo teve como objetivo central selecionar e validar proteínas candidata a marcadora de microambiente de leucemia infantil e realizar estudos funcionais e estruturais mais aprofundados com uma delas.

## B. Objetivos Específicos

**1. Seleção e validação de proteínas candidatas a marcadora de microambiente de leucemia infantil.**

**1.1.** Selecionar, a partir de dados prévios, proteínas candidatas a marcadora de microambiente de leucemia infantil preferencialmente de função e estrutura desconhecidos.

**1.2.** Verificar ativação trancripcional perante estimulo com células leucêmicas por qPCR a fim de validar os potenciais marcadores.

**2. Caracterização funcional e estrutural de uma proteína marcadora validada.**

**2.1.** Amplificação e clonagem do cDNA da proteína marcadora selecionado em vetores de expressão e em vetor específico para análise de duplo-híbrido em levedura.

**2.2.** Expressão e purificação da proteína para estudos estruturais (CD, SAXS, Cristalização / Difração de raios-X, etc.).

**2.3.** *Screening* através de duplo-híbrido em levedura a fim de mapear interações e obter pistas sobre a função da proteína.

# V. Resultados

## A. ARTIGO I:

# CHARACTERIZATION OF HUMAN STANNIOCALCIN-1 PROTEIN INTERACTIONS

Daniel M. Trindade, Marcos T. Santos, José A. Yunes & Jörg Kobarg

(Manuscrito em preparação)

# Characterization of human Stanniocalcin-1 protein interactions

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## Abstract

Stanniocalcin-1 (STC1) is an endocrine hormone originally discovered in bony fishes and later identified in humans were it seems to have a role in carcinogenesis and angiogenesis, although still unclear. The leukemia and solid tumors are dependent on vascularization and showed a significant increase in angiogenesis, estimated by the density of microvessels (DMV) demonstrating the important role of angiogenesis in the bone marrow in the pathogenesis and progression of hematological malignancies. Triggering of angiogenesis is a fundamental process to tumor-host interaction and essential to cancer progression. Some angiogenesis inhibitors showed effective anti-leukemia activity in animal models of human leukemia, indicating leukemia dependence on angiogenesis. Previous microarray analysis resulted in several activated genes in bone marrow (BM) endothelial cells (BMEC) modulated by leukemia presence. In view of the lack of information about molecular partners and STC1's actual role we present STC1 as a BM microenvironment molecular marker during acute lymphoblastic leukemia (ALL) validating previous microarray data by RealTime-PCR and performed yeast two hybrid screenings looking for some of the human STC1 proteins interactions providing a protein-protein interaction map, originally derived from the 22 interactants found on our yeast two-hybrid screenings. The proteins found are located in compartments where STC1 has been show to be present and might help to clarify how and which role it performs on these sites.

## Background

STC1 is an endocrine hormone originally discovered in bony fishes and later identified in humans by mRNA differential display of genes related to cellular immortalization, a key aspect of the cancer cell phenotype [1]. Different of its role in fish, human STC1 seems to play diverse roles in numerous developmental, physiological, and pathological processes including cancer, pregnancy, lactation, angiogenesis, organogenesis, cerebral ischemia, and hypertonic stress [2]. The precise role of STC-1 in carcinogenesis is still unclear, and in particular, the involvement of cancer cells in the differential STC-1 expression remains to be determined. STC-1 mRNA was present in the bone marrow and blood of breast cancer patients, whereas no STC-1 mRNA was

evident in healthy volunteers [3], suggesting STC-1 as a novel molecular marker for human breast cancer. Tumor vasculature may also be responsive for the increased expression of STC-1 [4;5]. The fact that VEGF induces STC-1 expression supports this assumption [6;7]. Studies also indicate that STC1 modulates inflammation, inhibiting mouse macrophages and human monoblasts chemotaxis [8] and decreasing macrophages and T-lymphocytes transmigration across endothelial cells [9]. Albeit STC1 has been implicated in so many processes, neither its receptor or other molecular partners nor its exact role in such processes are known yet.

Triggering of angiogenesis is a fundamental process to tumor-host interaction and essential to cancer progression [10]. Solid tumors, as well as leukemias, benefit from this new vascularization by obtaining oxygen, nutrients and growth factors. Some angiogenesis inhibitors showed effective anti-leukemia activity in animal models of human leukemia, indicating leukemia dependence on angiogenesis [11]. Leukemic cells are able to modulate its bone marrow (BM) microenvironment in its own benefit [12]. Microarray analysis (unpublished data), carried out in order to characterize leukemia-modulated genes in BMEC, by stimulation either with primary ALL cells or with the patient's plasma, resulted in several activated genes. While validating potential BM microenvironmental markers by Quantitative Real-Time PCR (qPCR) we found stanniocalcin-1 (STC1) as one highly activated gene in BMEC after 6 hours stimulus with plasma of children with ALL, as presented here. Also, in view of the lack of information about molecular partners and STC1's actual role we performed yeast two hybrid screenings looking for some of the human STC1 proteins interactions.

# Results

### Validation of STC1 as BM microenvironment molecular marker in ALL

Among proteins selected for validation are: ALL1 fused gene from chromosome 1q (AF1Q), CGI-109 which has been recently renamed to transmembrane emp24 protein transport domain containing 7 (TMED7), t-complex-associated-testis-expressed 1-like (TCTE1L or also known as dynein light chain Tctex-type 3 [DYNLT3]), dickkopf homolog 1 (DKK1), and STC1. All proteins had altered expression in previous microarray analysis but from those only two presented differential expression in our RealTime-PCR validation, being DKK1 expression downregulated and STC1 expression upregulated. Previous microarray data showed around 8 fold increase of STC1 on BMEC when treated with ALL plasma. We had confirmed STC1 mRNA up regulation during co-culture bone marrow estromal cells with leukemic blasts by RealTime Quantitative PCR (qPCR) and found a similar increase (~7 fold) in estromal cells co-cultured with 3 different patients ALL cells after 24 hours of treatment (Figure 1), unexpectedly after six hours of treatment STC1 mRNA was ~5.4 fold below its initial level (0h).

**Identification of STC1 interacting proteins by yeast two hybrid**

Before the yeast two-hybrid screening the construction of LexA-STC1ΔNterm was tested for autonomous activation of reporter genes (*HIS3* and *LacZ*) and no activation was detectable (not shown). We then employed yeast two hybrid system for screening of three human cDNA libraries: fetal brain, bone marrow and leucocyte. LexA-STC1ΔNterm construct was used as bait, and a total of about $1.5x10^6$ co-transformed clones in the three screenings were assayed for both reporters. A total of 22 proteins were identified and their retrieved fragments and domain composition as well as their cellular compartment/location according to GeneOnthology [13] are presented on Table 2.

Proteins were grouped according to its cellular compartment in seven groups, although some are present in more than one group: nuclear (LMNA, MAPK14, FUS, QRICH1, SAP18, SP100 and SUMO1), endoplasmic reticulum (ERN1, JSRP1, TMEM132A and FLJ20254), mitochondria (MTND1 and ALAS2), cytoplasm (ALDOA, FTL, MAPK14 and SUMO1), plasma membrane (ANPEP, CMTM3, DAGLB, FNDC4 and TMEM132A), extracellular/secreted (ELA2) and red blood cells (HBA1/2). Although QRICH1 and FLJ20254 had no GeneOnthology information about their location we used prediction of PSORT II program [14] to infer their possible localization.

**Confirmation of interactions by pulldown assay**

To confirm interactions observed in the yeast two hybrid screenings we performed a pulldown assay, using preys proteins (retrieved from the screenings) fused to a GST-tag and a His-tagged STC1 (STC1-HT) produced in insect cells (Trindade et al., submitted)[1]. However only eleven of them could be subcloned into GST-tag expression vector and, from those, only six presented soluble expression in bacteria (SP100, FUS, JSRP1, SUMO1, TMEM132A and LMNA), for FNDC4 protein primers were designed to amplify only the extracellular portion (mainly comprising its fibronectin type III domain[FNIII]) excluding its carboxyl-terminal end (transmembrane and intracellular portion). Recombinant STC1-HT was capable to bind to all seven GST-tagged proteins but not with GST alone demonstrating the specificity of the interaction (Figure 3).

**Mapping interactions**

We further generated two constructs of STC1 fused to LexA DNA binding domain splitting STC1 in two halves (an amino and a carboxyl end construct, named LexA-N STC1ΔPS and LexA-

[1] Trindade, D.M.; Silva, J.C.; Navarro, M.S.; Torriani, I.C.L; Kobarg, J. *Low-resolution structural studies of human Stanniocalcin-1*. Submitted for publication on March 2009.

C STC1) in the only region in which disulfide bonds didn't cross in the primary sequence (Figure 2), and both constructs were tested against prey constructs retrieved from yeast two hybrid screenings (Figure 4). Some proteins did not interacted with any of the partial constructs of STC1 (ANPEP, FLJ20254, FTL, HBA1/2, MTND1 and SUMO1), all others interacted with the amino terminal of STC1 but not with the carboxyl terminal end, except that TMEM132A presented higher interaction with N-terminal end than with the full length construct and FNDC4 interacted with both ends of STC1. Interestingly, in previous SAXS experiment (Trindade *et al.*, *submmited*) we had speculated that STC1 forms an anti-parallel dimer by its carboxyl terminal end leaving both its amino-termini exposed, which might, at least in part explain this result. During the mapping of interaction of ZBTB16 on STC1 N and C terminal constructs the prey also presented β-galactosidase activity with LexA-only construct, perhaps because it has been a transcription regulator [15;16], and thus was disconsidered from the mapping.

# Discussion

Although STC1 is becoming more and more evidently involved in many cellular processes, its molecular partners have not yet been exploited or characterized. This is the first work looking after protein interactions of STC1 in order to better understand its roles in this variety of cellular processes. Among all retrieved interactants there are proteins from different compartments as nucleus, cytoplasm, mitochondria, endoplasmic reticulum (ER) / Golgi complex, plasma membrane and also extracellular ones. Despite STC1 has been characterized to be a secreted hormone, that is present in all secretory compartmens from ER through Golgi complex and extracellular space, it has also been shown to have high affinity 'receptors' at the plasma and mitochondria membranes [17], and was found at mammary gland alveolar cells nuclei [18]. Following we will address the possible implications of STC1 in each of the compartments found.

### Nuclear proteins

The interphase nucleus is a highly compartmentalized organelle, in which chromosomes occupy discrete territories and various regulatory proteins are present in specific nuclear bodies and/or are diffusely distributed throughout the nucleoplasm [19]. The lamins, which belong to the intermediate filament family of proteins, are the major components of a filamentous network underlying the inner nuclear membrane, termed the nuclear lamina. Among roles attributed to lamins are maintenance of nuclear envelope integrity, chromatin anchoring sites supply and determination of interphase nuclear architecture. Lamins A/C (encoded by LMNA gene) seems to

have a role in spatial organization of the speckles, one of the ever-increasing number of nuclear bodies, where it seems to organize RNA splicing factors and polymerase II transcription [20-22].

FUS, also known as TLS, is member of the FET family of RNA binding proteins, which also includes EWS and TAF15 (TAFII68, TAF2N, RBP56) proteins that had been found in the nucleus as well as in the cytoplasm and shown to shuttle between these locations. FET proteins are targeted to stress granules induced by heat shock and oxidative stress and FUS required its RNA binding domain for this translocation [23]. Also, the small nuclear ribonucleoprotein (snRNP)-associated protein of 69 kDa (69KD), a protein that shares structural similarity with members of FET (TLS and EWS, 95% and 65% identity, respectively), has been shown to be present in speckles [24]. Interestingly FUS was one of the most abundant proteins retrieved in our STC1 yeast two hybrid screenings. STCs are implicated stresses situations like hypoxia [25] and oxidative stress [26], so its likely that it could be found in stress granules together with FUS.

Another nuclear subdomain are the promyelocytic leukemia (PML) nuclear bodies (NBs) which recruit and locally accumulate an amazing number of proteins, many of which are key regulators of various processes. PML is present both in the nucleoplasm and in NBs, which also are nuclear matrix associated [27], and play a role in the organization of these NBs, targeting proteins such as Sp100, CBP, or Daxx onto these domains [28]. Sumoylation was first proposed to target PML toward NBs, behaving as an NB-targeting signal but now sumoylation is seen clearly not an NB-targeting signal, but maybe a consequence of the NB localization, although the functional significance of the presence of so many sumoylated proteins in NBs remains to be understood [27;28]. Interestingly, most of PML-NB proteins are transiently residents and are recruited or released upon different cellular stress signals. An example is p53 protein that during many kinds of stress is stabilized and activated at the PML-NB and then can induce the transcription of its targets genes leading to cell cycle arrest, senescence or apoptosis [29;30]. Also, exposure of cells to high concentrations of heavy metal affects SP100 and PML proteins of PML-NB altering their number and distribution within the cell [31]. Although ZBTB16 has been disconsidered during mapping experiments it also seems to colocalize with PML-NBs [32] and may bind to STC1 direct or indirectly. Some PML proteins (e.g. Daxx, PIASx, HIPK2, Topors), had been shown to have a SUMO interacting motif (SIM) [33] providing a non-covalently bind to SUMO enable binding to other sumoylation proteins and consequently affecting its cellular location. As presented here STC1 interacted both with SP100 and SUMO1, so we speculate that STC1 would have a structural SIM. In addition STC1 presented in its sequence three putative sites for sumoylation (Trindade et al., *submitted*)[1]. Another evidence linking STC1 to PML-NB is that during cell death generated by iodoacetamide-induced oxidative stress, p53 is stabilized, acetylated and furthermost activates STC1 expression [34]. Also, Law and co-workers [35], using trichostatin A (an inhibitor of Histone deacetilases [HDACs]), reported hyper acetylation of

STC1 promoter region, together with the recruitment of NFκB, to be involved in the activation of STC1 gene expression.

Another STC1 yeast two hybrid retrieved protein, SAP18 (Sin3-associated polypeptide of 18 kDa), has been shown to play a key role in gene-specific recruitment of the HDAC complex by a number of transcription factors, acting as a protein-protein adapter module bridging the Sin3-HDAC complex to transcription factors, which is supported by its ubiquitin-like fold [36;37]. Recently HDAC7 has been shown to act as a SUMO E3 ligase promoting PML sumoylation, in a deacetilase independent fashion, and playing an important role in regulation of PML-NB formation [38;39].

**Endoplasmic reticulum proteins**

Among the four ER retrieved proteins two are related to unfolded protein response (UPR): the UPR transducer ERN1; TMEM132a, which had been renamed to HSPA5 binding protein (HSPA5BP) by its high homology to its rat ortholog [40]. The UPR is activated by a variety of insults that disrupt protein folding in the ER lumen thus preventing accumulation of unfolded proteins in the ER lumen. Among these insults are changes in intralumenal calcium, altered glycosylation, nutrient deprivation, pathogen infection, expression of folding-defective proteins, and changes in redox status [41]. The mammalian UPR has been shown to be activated by three interconnecting signaling proteins: activating transcription factor 6 (ATF6), IRE1 (first identified in a yeast mutant with inositol-requiring phenotype, and also known as ERN1), and double-stranded RNA-activated kinase (PRK)-like ER kinase PERK. Each of these proteins is localized to the ER membrane bounded to glucose-regulated protein 78 (Grp78, also known as BIP and HSPA5), a soluble ER-resident molecular chaperone, that upon ER stressing conditions is released from the trio of UPR transducers [42]. Even more interesting is that the other member of STC family of proteins, STC2, has been shown to be induced by ER and oxidative stress agents, and its knockdown accentuated N2a cell death induced by thapsigargin, an inhibitor SERCA (sarco / endoplasmic reticulum $Ca^{2+}$ ATPase) via UPR [26].

The other two ER retrieved proteins are the junctional sarcoplasmic reticulum protein 1 (JSRP1), and the FLJ20254 protein (recently named TMEM214) of unknown function. JSRP1 plays a modulatory role in calcium turnover in muscle, as shown by its interaction to both, dihydropyridine receptor (DHPR) voltage sensors and, inside sarcoplasmic reticulum's lumen binds to calsequestrin which in turn is linked to Ryanodine receptors family of calcium release channels of the reticulum [43;44]. In fish STC1 has been well characterized to play roles in calcium and phosphate homeostasis [45-47]. As for human STC1 there is a Japanese group that has shown

evidences for STC1 in stress related to calcium-overload [48-50] so we may speculate that STC1 might inhibit JSRP1 by interacting with it.

**Mitochondrial proteins**

STC1 has been previously found confined within the mitochondrial matrix, having concentration-dependent stimulatory effects on NADH oxidation [17] which was shown to be attenuated upon occupancy of the ATP binding cassette by purine nucleotides such as ATP and GTP [51]. STC1 effect on the electron transport chain could be mediated by its binding to MTND1, possibly enhancing its activity. Other proteins related to electron transport chain were initially retrieved from the STC1 yeast two hybrid screen (data not shown) but when co-transformed with STC1 did not confirmed to be directly interacting. However their retrival in the first place may have occurred in the form of a complex and there fore reinforces the presence of STC1 in these protein complexes.

**Red blood cell and secreted proteins**

The second more abundant protein retrieved from the STC1 yeast two hybrid screens were the two hemoglobin beta chain genes (HBA1 and HBA2), which code for the same protein. Circulating STC1 is usually not detected, except in pregnancy [2], however James and coworkers [52] demonstrated high binding of STC1 to red blood cells postulating that the inability of STC1 detection in serum could be due its sequestration by red blood cells which either could represent a mechanism of delivery or removal of STC1 from blood and in both cases HBA1/2 could serve as this unknown capture protein. Another erythroid related protein found was 5-Aminolevulinate synthase 2 (ALAS2) one of the existing two ALAS isozymes that catalyzes the first and rate-limiting step of heme synthesis, and in the case of ALAS2, which is expressed exclusively in erythroid cells is responsible for the synthesizes of heme specifically for haemoglobin [53]. Interestingly ALAS2 transcription is hypoxically induced in an HIF-1–independent manner, leading to an increase in heme content [54].

**Membrane proteins**

Aminopeptidase N (ANPEP or also called CD13) is a broad specificity aminopeptidase that plays a role in the final digestion of peptides generated from hydrolysis of proteins by gastric and pancreatic proteases. In addition ANPEP serves as receptor for human conavirus and cytomegalovirus infections, as well as for tumor-homing peptides, more specifically NGR peptides, and has also been pointed as an important regulator of endothelial morphogenesis

during angiogenesis [55;56]. Chemokine-like factor superfamily 3 (CKLFSF3, or also known as CMTM3 which stands for CKLF-like MARVEL transmembrane containing motif protein 3), belongs to a novel family of proteins that plays important roles in the immune system and participate in tumorigenesis, in particular, CMTM3 is highly expressed in the testes along with leukocytes and placenta [57;58]. Fibronectin domain containing protein 4 (FNDC4 or FRCP1) is strongly expressed in brain and liver, and like fibronectin type III (FNIII) and Arg-Gly-Asp (RGD) containing plasma membrane proteins might serve either as cell adhesion molecules or as receptors with no identified ligant. Taking into account that STC1 does not have a characterized receptor it would be interesting to perform further experiments in order to confirm that any of these candidates would fulfill the role for STC1 receptor and current work is being done in that direction by us.

**Other (Cytoplasmatic) proteins**

Mitogen-activated protein kinase 14 (MAPK14 or p38αMAPK) is a key kinase that responds to activation by environmental stress and pro-inflammatory cytokines by phosphorylating a number of transcription factors and several downstream kinases. Another branch of the mitogen activated protein kinases are the extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), which are also known as the classical mitogen kinase cascade, acting in signaling cascades that regulates various cellular processes such as proliferation, differentiation, and cell cycle progression [59]. Holmes & Zachary [60] recently proposed that STC1 expression induced by VEGF-A165 would be mediated primarily by PKC, ERK and calcium signalling pathways. We could speculate that STC1 might then act in a modulatory fashion inhibiting the stress-related via of MAPK by binding to its most abundant isoform, p38αMAPK (MAPK14).

Frutose-biphosphate aldolase A (ALDOA) have been show to be activated by HIF-1α [61], as well as STC1 [25], and recently activation of an HIF-1α-regulated glycolysis have been suggested to be related to the aggressive phenotype of cancer [62].

Although Sn1-specific diacylglycerol lipase beta (DAGLB) is a transmembrane protein the majority of it is oriented to the cytoplasm, so it will be analyzed here. DAGLB catalyzes the hydrolysis of diacylglycerol (DAG) to 2-arachidonoyl-glycerol (2-AG), the most abundant endocannabinoid in tissues, being stimulated by glutathione and calcium [63].

In Conclusion our yeast two hybrid screenings provided a first glance to what might be a clearly multifunctional protein as shown by the protein interaction map (Figure 5). Although future and more detailed studies must be performed in order to clarify the pathways and which role STC1 plays in all these processes this first study in this scale to date shed some light in the function of this protein.

# Methods

**Co-culture Assay for validation of molecular marker candidate diferential expression in BM stromal cells (Validation of microenvironment markers)**

In order to validate previous microarray data we performed co-culture of BM stromal cells with leukemic blasts. BM stromal were cultured in DMEM media with 10% FCS in six-well plates until reach around 70% of confluency, at this point the media was changed either to fresh media (control) or fresh media containing 3x106 leukemic blasts (three patient samples were used in parallel experiments). Samples from non-treated BM stromal cells (0h), or treated with fresh media containing, or not, leukemic blasts (Figure 1A). Cells were washed with HBSS 1x in order to separate leukemic blasts (non adherent) from adherent stromal cells, which were then trypsinized and the total RNA extracted.

**Real-Time Quantitative PCR (qPCR)**

For qPCR, total RNA was extracted from samples of treated or untreated stromal cells using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol and quantified by spectrophotometrical methods.

Two micrograms of total RNA was treated with DNase (GE Healthcare) and then transcribed into first strand cDNA using First Strand cDNA Synthesis Kit (Amersham Biosciences / GE Healthcare) modified according to previously described [64]. Briefly, RNA was treated with 20U of DNase I (Amersham Biosciences / GE Healthcare) in Tris-HCl 40 mM pH 7.5, MgCl2 6 mM buffer, after 15 minutes DNase was inactivated by heating at 80°C for 10 minutes. For the cDNA synthesis, we proceeded similar to manufacturer's protocol except that 500ng of random hexamers were used.

For qPCR assays were performed using the Applied Biosystems 7500 Systems (Applied Biosystems) and each sample was run in triplicate to ensure quantitative accuracy. All PCR reactions were carried out in a final volume of 25µl containing 1X of SYBR Green PCR Master Mix (Applied Biosystems), a previously determined concentration of each gene specific primers (Table 1), 1 µL stromal cell cDNA, and sterile deionized water. The standard cycling condition was 50 °C for 2 min, 90 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analysed on 7500 System SDS software (version 1.3) by relative quantification ($2^{-\Delta\Delta Ct}$) using GADPH as endogenous control and non-treated BM stromal cell cDNA as calibrator sample.

**Plasmid Constructs**

In order to obtain all STC1 constructs we used pGEM-STC1 (Trindade *et al.*, *in press*) construct as template and PCR amplified products were cloned into pUC18 or pGEM plasmids, as follows: for pUC18-STC1 ΔNterm (STC1 lacking the first 22 aminoacids) were used STC1 ΔNterm F (5' aaggatccCAGAATGACTCTGTGAGCCC 3') and STC1 R (5' ccgaattCCTCTCCCTGGTTATGCAC 3') primers and the PCR product inserted into pUC18 SmaI site in order to obtain two EcoRI sites (one from the insert and another from the vector) on each end of the insert; for pGEM-N STC1 ΔPS (STC1 N terminal fragment lacking the signal peptide sequence, residues 18 to 134) and for pGEM-C STC1 (C terminal of STC1 consisting of residues from 129 to 247) were used the following primers pairs STC1ΔPS F2 (5' gaattcACCCATGAGGCGGAGCAG 3') and

NSTC1+stop R2 (5' ggatcc**tta**CACATTCAGCTTGCTGTAG 3), CSTC1 F2 (5' gaattcTACAGCAAGCTGAATGTGTG 3') and CSTC1 R2 (5' ggatccTTATGCACTCTCATGGGATG 3'), respectively. Capital letters indicate sequence identical to STC1 cDNA, small caps letters indicate sequence non-identical to template. All pGEM and pUC18 constructs were verified by DNA sequencing in order to ascertain the correct nucleotide sequence. pUC18-STC1ΔNterm was digested with EcoRI and both pGEM-N STC1 ΔPS and pGEM-CSTC1 were digested with EcoRI and BamHI and these inserts were cloned into the same sites of pBTM116 resulting in a protein fused with LexA DNA binding domain (Figure 1).

In order to obtain constructs used on the pull down assay all nucleotide sequences encoding the proteins identified to interact with the STC1, except that encoding FNDC4 , were subcloned from the vector pACT2 to the modified bacterial expression vector pET28a-GST-Tev [65] resulting on GST fusion recombinant proteins constructs which were all verified by DNA sequencing in order to ascertain the correct nucleotide sequence. On the other hand FDNC4 sequence was PCR amplified from the pACT2 vector with the following specific primers (5' aaggatccCGGCCTCCCTCTCCTGTG 3' and 5' gggaattcACTCAAACGTCGATGGTGTTG 3') and the obtained construct [GST-FNDC4(FIII)] lacks the original carboxyl terminus portion which encodes its transmembrane and cytoplasmic domains.

**Yeast Two-hybrid Screen and DNA Sequence Analyses**

The yeast two-hybrid screenings of human cDNA libraries (Clontech) from fetal brain, bone marrow and leukocytes were performed using the yeast strain L40 (trp1-901, his3del200, leu2-3, ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lac GAL4) and STC1ΔNterm as a bait in

pBTM116 vector. L40 were transformed according to the protocols supplied by Clontech and *HIS3* and *LacZ* reporters were assayed as previously described [66].

The screening was performed on Synthetic Dropout minimal medium plates (SD) without tryptophan, leucine, and histidine according to the library screening protocols supplied by Clontech. Recombinant pACT2 plasmids of positive clones for both reporters were isolated and their inserts sequenced with a DNA ABI PRISM 377 Genetic Analyser (Applied Biosystems). The obtained DNA sequence data were annotated using Basic Local Alignment Search Tool (BLAST) web interface [67] and clones aligning to genomic contigs or untranslated regions were discarded.

**Protein Expression, Purification and Pulldown assay**

Bacterial expression of yeast two hybrid retrieved proteins was obtained expressing GST fusion protein constructs in Escherichia coli BL21 (DE3) pRARE cells at the following conditions: (1) 37 °C using 0.5 mM isopropyl 1-thio-B-D-galactopyranoside (IPTG) for 4 h [GST-FNDC4(FIII), GST-JRSP1 and GST-SUMO1], and (2) 25 °C using 10mM lactose for 16 hours [GST-FUS, GST-LMNA, GST-SP100 and GST-TMEM132A]. For each construct 25mL of transformed E coli BL21(DE3) pRARE culture were harvested by centrifugation at 4,500x g for 10 min, and the cell pellet was resuspended and incubated for 1 hour on ice in 1mL of lysis buffer (PBS [137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4] containing 0.2 mg/mL lysozyme, 1 µg/mL DNaseI, 1 µg/mL RNAse and 6 mM MgCl2) and lysed by sonication. Samples were centrifuged at 20,000x g for 30 min at 4 °C and the supernatant used as soluble total lisate. STC1-HT was expressed in insect cells and purified as previously described (Trindade, et al., *submitted*).

One milliliter of total lisate of all GST tagged constructs or of GST alone was allowed to bind to 25 µL of Glutathione-Uniflow Resin (Clontech) for 1 h at 4 °C. After incubation, the beads containing bound recombinant proteins were washed three times with ice cold PBS. Twenty five micrograms of purified STC1-HT protein were added to the resins containing GST fusion proteins or GST alone and further incubated in 0.1 mL of PBS for 4h at 4 °C to allow protein-protein interactions to occur. Beads were then washed three times with 0.5 mL of PBS, followed by three washes with 0.5 mL of PBS containing 0.2% Triton X-100, then three washes with 0.5 mL of PBS without Triton X-100. Resin-bound protein complexes were resolved on two separate 12,5% SDS-polyacrylamide gels and after electrophoresis, the proteins were transferred to PVDFmembranes by semi-dry electroblotting. Membranes were blocked with 5% BSA in TTBS (0.15M NaCl, 20mM Tris-HCl, 0.05% Tween-20, pH7.2) for 1 hour, then incubated either with a mouse anti-His tag (1:5000; QIAgen) or mouse monoclonal anti-GST [68] in blocking solution

for 1 h. After three washes with TTBS membranes were incubated with the HRP-conjugated anti-mouse antibody (1:5000; Santa Cruz Biotechnology) for 1 h and washed again three times with TTBS. Blots were developed by Luminol reagent (Santa Cruz).

**Protein-protein interaction network construction**

Osprey program [69] was used either for STC1 interaction network construction as well as on already known interactions by search on the Biological General Repository for Interaction Datasets (BioGRID) [70;71].

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# Tables

**Table 1 - Primers sequences and concentrations used at the qPCR**.

| GenBank Accession Number | Primer name | Primer Sequence (5´-3´) | Product size (bp) | Concentration in qPCR reaction (nM) |
|---|---|---|---|---|
| NP_006809 | AF1Q-F<br>AF1Q-R | GGCCTGGGTCTGTCAGATACA<br>TGCTTGCCCGATCATTTTG | 72 | 400 |
| NP_861974 | CGI109-F<br>CGI109-R | AGGGAGGAAGAAAATTGCCTTT<br>TCCATAAAACTGAACAGTGCAGAATA | 95 | 400 |
| NP_036374 | DKK1-F<br>DKK1-R | AGGAAGCGCCGAAAACG<br>TTTGATCAGAAGACACACATATTCCA | 88 | 400 |
| NP_003146 | STC1-F<br>STC1-R | TGAGGCGGAGCAGAATGACT<br>CAACGAACCACTTCAGCTGAGT | 78 | 100 |
| NP_006511 | TCTE1L-F<br>TCTE1L-R | ATGCTGAGGAAGCCCACAAT<br>TTATAGGCTTTTCCCAACTTAACCA | 148 | 250 |
| NP_002037 | GAPDH-F<br>GAPDH-R | ATGGAAATCCCATCACCATCTT<br>CAGCATCGCCCCACTTG | 68 | 400 |

**Table 2 - Proteins retrieved from yeast two hybrid screenings**

| cDNA Library (n°. of clones) | Official Simbol (Entrez GeneID) [72] | Protein Fragment | Domain(s) present | Component/Compartment according to GeneOntology [13] |
|---|---|---|---|---|
| BM (9) | FUS (2521) | 381-526 (526) | ZnF RBZ | - nucleus<br>- membrane<br>- cellular component unknown |
| BM (8) | HBA1/2 (3039/3040) | 1-142 (142) | G | - hemoglobin complex |
| BM (5) | JSRP1 (126306) | 1-314* (331) | | -undetermined |
| BM (4) | FLJ20254 (54867) | 601-689 (689) | DUF2359 | -undetermined |
| L (4) | SAP18 (10284) | 81-153 (153) | | - histone deacetylase complex |
| FB (1) & L (1) | MT-ND1 (4535) | 9-60 (318) | NADHdh | - mitochondrial respiratory chain complex I<br>- integral mitochondrial inner membrane |
| FB (2) | FNDC4 (64838) | 15-234 (234) | FN3 | - integral to plasma membrane |
| L (2) | ERN1 (2081) | 1-59 (977) | PQQ | - integral to endoplasmic reticulum membrane |
| L (2) | CMTM3 (123920) | 34-182 (182) | | - extracellular space<br>- integral to plasma membrane |
| L (2) | MAPK14 (1432) | 1-38 (360) | | - cytoplasm<br>- nucleus<br>- spindle pole |
| L (2) | ANPEP (290) | 875-967(967) | | - cytoplasm<br>- integral to plasma membrane<br>-ER-Golgi intermediate compartment |
| BM (2) | ELA2 (1991) | 190-267 (267) | Tryp SPc | - cell surface<br>- extracellular region |
| L (2) | SP100 (6672) | 704-852 (879) | HMG, CC | - nucleus<br>- PML body<br>- chromatin / chromosome |
| BM (1) | LMNA (4000) | 439-572 (572) | IF tail | - nucleus<br>- nuclear lamina intermediate filament |
| BM (1) | ALDOA (226) | 89-364 (364) | FBP AIa | -undetermined |
| BM (1) | FTL (2512) | 1-175 (175) | | - ferritin complex |
| BM (1) | ZBTB16 (7704) | 55-176 (673) | BTP | - nucleus<br>- nuclear speck<br>- PML body<br>- transcriptional repressor complex |
| BM (1) | ALAS2 (212) | 341-460* (587) | AAT I PLP | - endoplasmic reticulum<br>- mitochondrial matrix<br>- integral to mitochondrial inner membrane |
| L (1) | QRICH1 (54870) | 502-667* (776) | | - intracellular |
| L (1) | SUMO1 (7341) | 1-101 (101) | UBQ | - cytoplasm<br>- membrane<br>- nucleus<br>- nuclear pore<br>- nuclear membrane<br>- nuclear speck |
| FB (1) | TMEM132A (54972) | 808-932* (1024) | CC | - endoplasmic reticulum<br>- Golgi apparatus<br>- integral to endoplasmic reticulum membrane membrane |
| L (1) | DAGLB (221955) | 613*-668 (672) | | - integral to plasma membrane |

**BM:** Bone Marrow; **FB:** Fetal Brain; **L:** Leucocyte; **AAT I PLP:** Aspartate aminotransferase superfamily (fold type I) of pyridoxal phosphate; **BTP:** Bromodomain transcription factors and PHD domain containing proteins; **CC:** coiled coil; **DUF2359:** Uncharacterised conserved protein; **FBP AIa:** Fructose-1,6-bisphosphate aldolase Ia; **FN3:** Fibronectin type III; G: Globin; **HMG:** high mobility group; **IF tail:** Intermediate filament tail; **NADHdh:** NADH dehydrogenase; **PQQ:** Beta-propeller repeat occurring in enzymes with pyrrolo-quinoline quinone; **Tryp SPc:** Trypsin-like serine protease; **UBQ:** Ubiquitin homologues; **ZnF RBZ:** Zinc finger domain in Ran-binding proteins.

# Figures

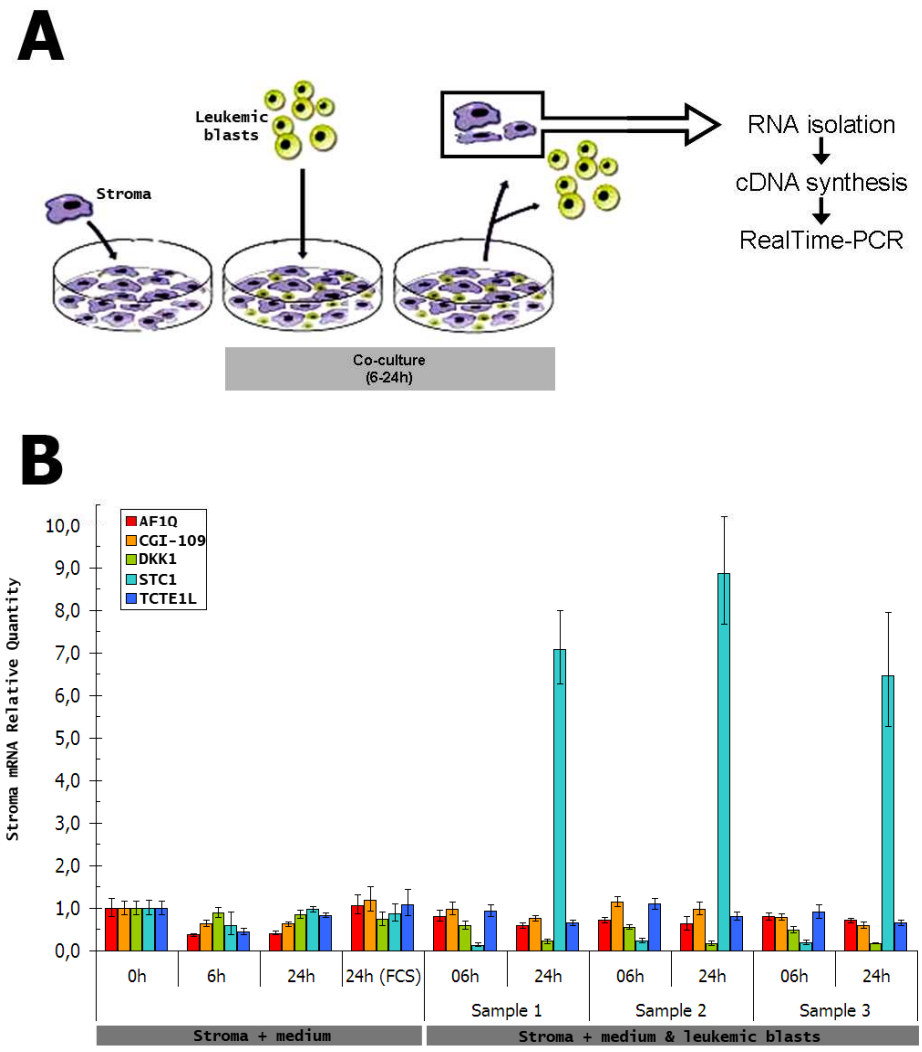


**Figure 1. Analysis of candidate gene expression by Quantitative Real-Time PCR (qPCR).**
**(A)** BM stromal cells were cultured until reach confluence and then subjected to co-culture with leukemic blast from different patients for different periods of time. BM stromal cells were separated and processed for RNA extraction, cDNA synthesis and RealTime Quantitative PCR (qPCR). **(B)** mRNA expression of different candidate genes in BM stromal cells either cultured in fresh media without fetal calf serum (FCS) during 6 hours and 24 hours (both with or without FCS), or stimulated by co-culture with primary leukemic blasts of three different ALL patient's (Sample 1, 2 and 3) in fresh media without bovine fetal serum (BFS). The same sample of BM stromal cells before experiments (0h) was used as calibrator. Bars indicate standard error with 95% of confidence. DKK1 presented reduction in expression level as STC1 presented initially reduction, after 6 hours of co-culture, and than, after 24 hours, a great increase in expression level.

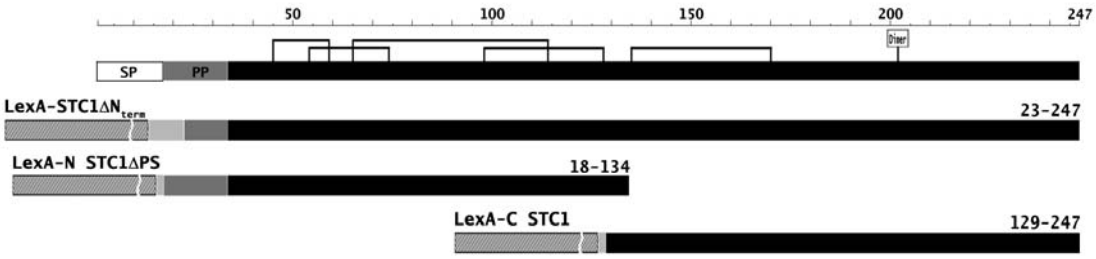


**Figure 2. Schematic representation of full-length human STC1 and different constructs with LexA DNA binding domain herein used.**
It is shown a linear representation of STC1-HT amino acid sequence with assignment of its different portions (signal peptide [SP] in white, pro-peptide [PP] in dark gray, and mature protein chain in black), and its disulfide bonds intra- (brackets) and inter-chain (Dimer box). All constructs produced chimeric proteins with LexA DNA binding domain (LexA) the one coding for LexA-STC1$\Delta N_{term}$ was used as bait in the yeast two-hybrid and all three constructs were used for mapping interactions in yeast. Light gray bar indicates a linker region.

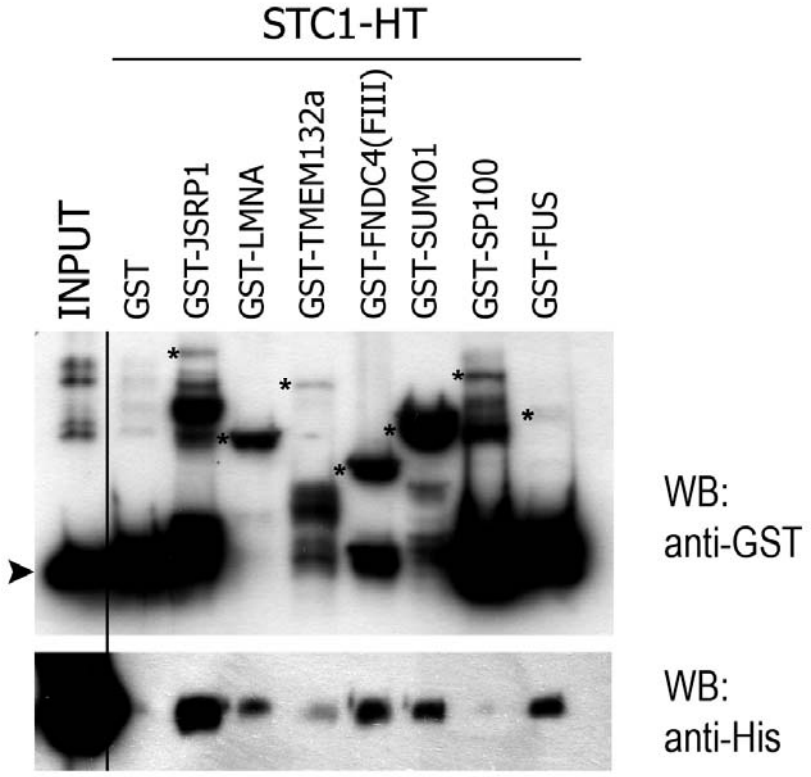


**Figure 3. *In vitro* confirmation of STC1 and selected retrieved proteins interactions.**
Pull down assay between STC1-HT produced in baculovirus system and fragments of selected prey proteins retrieved from yeast two-hybrid screening. All preys are GST fusion recombinant proteins. Asterisks indicate expected GST fusion recombinant protein sizes, arrow head indicates GST size. INPUT indicates GST input in the upper western-blot (WB: anti-GST) and STC1-HT in the lower western-blot (WB: anti-His).

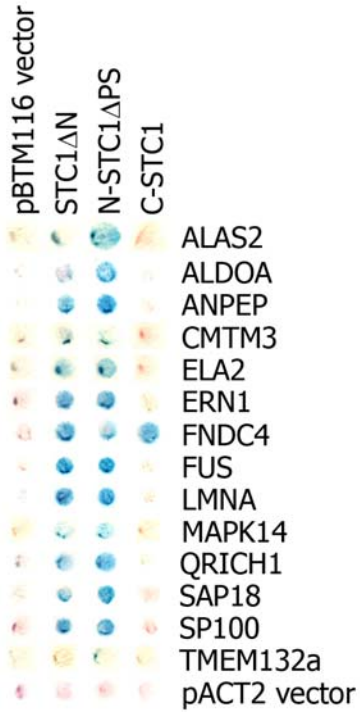


**Figure 4. Mapping of STC1 interactions by yeast two-hybrid.**
Colony lift β-galactosidase assay of yeast L40 strain co-transformed with pBTM116 constructs expressing LexA DNA binding domain fusion baits (top) and Gal4 activation domain fusion preys (right). Proteins that only interacted to STC1ΔN constructs are not presented here.

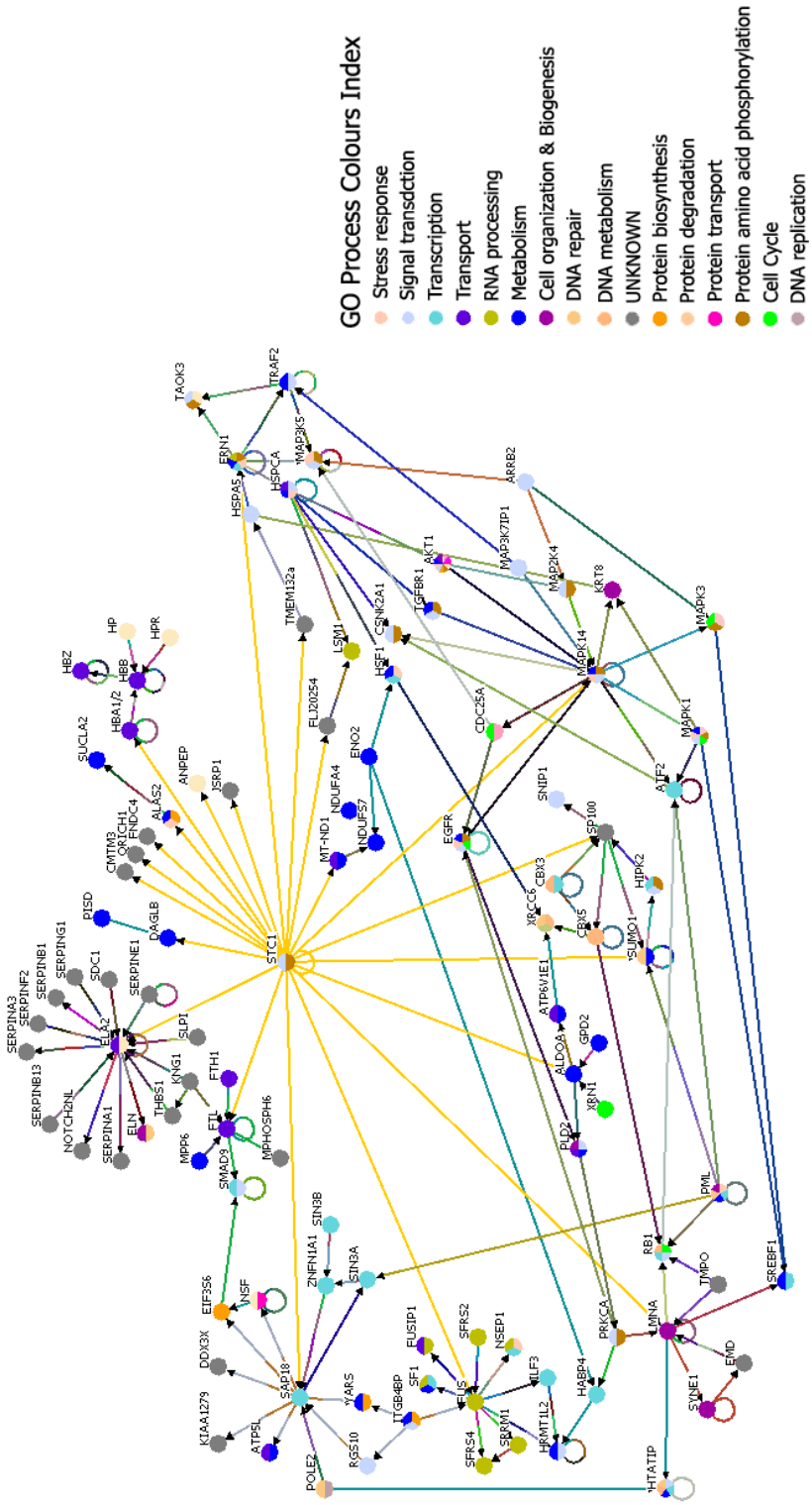


**Figure 5. Yeast two hybrid interaction map of human STC1 against Bone Marrow, Leucocyte and Fetal brain.**

Diagram showing the simplified network of proteins found by two hybrid screenings. Lines indicate interactions and each color is indicative of published data about the interaction (present work findings indicated yellow connecting STC1 to its preys, except ZBTB16). Each protein is represented by a different color assigned by Osprey program [69] according to GeneOnthology.

B. ARTIGO II:

# LOW-RESOLUTION STRUCTURAL STUDIES OF HUMAN STANNIOCALCIN-1

Daniel M. Trindade, Júlio C. Silva, Margareth S. Navarro, Iris C. L. Torriani & Jörg Kobarg

(Artigo Submetido à revista BioMed Central Structural Biology)

# Low-resolution structural studies of human Stanniocalcin-1

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## Abstract

**Background:** Staniocalcins (STCs) represent small glycoprotein hormones, found in all vertebrates, which have been functionally implicated in Calcium homeostasis. However, recent data from mammalian systems indicated that they maybe also involved in embryogenesis, tumorigenesis and in the context of the latter especially in angiogenesis. Human STC1 is a 247 aminoacids protein and a predicted molecular mass of 27 kDa, but preliminary data suggested its di- or multimerization. The latter in conjunction with alternative splicing and/or post-translational modification gives rise to forms described as "big STC", which range from 56 to 135 kDa. **Results:** In this study we performed a biochemical and structural analysis of STC1 with the aim of obtaining low resolution structural information about the human STC1, since structural information in this protein family is scarce. We expressed STC1 in both E. coli and insect cells using the baculo virus system with a C-terminal 6xHis fusion tag. From the latter we obtained reasonable amounts of soluble protein. Circular dichroism analysis showed STC1 as a well structured protein with 52% of alpha-helical content. Mass spectroscopy analysis of the recombinant protein allowed to assign the five intramolecular disulfide bridges as well as the dimerization Cys202, thereby confirming the conservation of the disulfide pattern previously described for fish STC1. SAXS data also clearly demonstrated that STC1 adopts a dimeric, slightly elongated structure in solution. **Conclusions:** Our data reveal the first low resolution, structural information for human STC1. Theoretical predictions and circular dichroism spectroscopy both suggested that STC1 has a high content of alpha-helices and SAXS experiments revealed that STC1 is a dimer of slightly elongated shape in solution. The dimerization was confirmed by mass spectrometry as was the highly conserved disulfide pattern, which is similar to that found in fish STC1.

## Background

Stanniocalcins (STCs) represent a small family of secreted glycoprotein hormones consisting of STC1 and STC2 in which amino acid sequences are highly conserved among aquatic and terrestrial vertebrates [1-7]. However, the lack of homology with other known proteins has

hampered the understanding of their functions. Initial evidence suggested that mammalian STC1 would parallel the function of fish STC1, which has been implicated in mineral homeostasis [8-10]. It is tempting to assume that the functions of STC1 and STC2 overlap at least in part, since they share high similarity in their primary amino acid sequence especially at the N-terminus and the pattern of cysteine residues is completely conserved [11].

However, there are also several differences between STC1 and STC2, including the fact that STC2 has 55 additional amino acids, the majority of which are located at its C-terminus [12-14]. Furthermore their expression patterns are different[1,14-17] and STC2 is unable to displace STC1 from its putative receptor [18,19], indicating that both molecules may have distinct receptors.

Although STC1 functions as an anti-hypercalcemic hormone in fish [20-22], it is becoming increasingly clearer that it may have expanded roles in mammals. Such assumption is based on its wide expression pattern in adult normal tissues[1,16,23-27], tumors [17,28,29] and also during embryogenesis [30-35]. Further support for a complex function of STC1 in mammals comes from studies that show its varying sub- cellular localizations [18,19] and of a gain-of-function phenotype observed in transgenic mouse [36,37].

Relatively little is also known about STCs molecular structure. The human and mouse genomes encode a 247 amino acid STC1 protein [17,38]. The first 204 amino acids show 92% sequence similarity to salmon STC1 and include a conserved N-linked glycosylation site of the type Asn-X-Thr/Ser (N-X-T/S) [17,39]. Compared to the fish STC1 however, the last 43 residues at the C-terminus are poorly conserved in human STC1 (and STC2), suggesting that the main biological activity of the STCs is mediated through its N-terminus [40,41].

In ancient fish, the last conserved cysteine residue in the C-terminal of STC1, which is supposedly involved in its dimerization, is replaced by arginine or histidine residues, thereby giving rise to a strictly monomeric form of the protein [42,43]. Although dimeric forms of STC1 have been described [39,44,45] answers to the question of its potential multimerization and modification to diverse higher molecular weight forms under certain circumstances remain elusive.

STC1 however, seems to exist in two different forms, the conventional dimeric 56 kDa form, consisting of two ~28 kDa monomers, also known as $STC_{50}$, and a number of higher molecular weight STC variants, collectively referred to as "big STC" [19,25,46-49]. At least three molecular weights: 84, 112, and 135 kDa have been described and big STC1 has been reported to be expressed in adipocytes, adrenocortical cells [47,48] and ovaries [19,25,49]. In order to explain the increased mass of big STC1 it has been suggested that either distinct post-translational modifications, including glycosylation [17,25] or phosphorylation [50]

occur in big STC or additional but yet uncharacterized exons [48] are being employed. In agreement with the latter, the monomeric form big STC1 is about ~10 kDa larger than the theoretically predicted monomer. Another possibility is the formation of tri- (84 kDa), tetra- (112 kDa) or even pentamers (140 kDa) of STC1, although in this case obtained values only add up for the theoretically predicted monomer (~28 kDa) but not for that observed for the big STC monomer (~38 kDa). It is however noteworthy that the 135-kDa variant of big STC1 found in adrenocortical cells is resistant to chemical reduction, just like $STC_{50}$ from the mitochondrial matrix [48], thereby suggesting the formation of a more stable and maybe durable quaternary structure.

In this paper we present structural information about the human STC1 protein. We expressed human STC1 in insect cells using a bi-cistronic baculovirus construct. After affinity purification we collected SAXS data for STC1 in solution. Data analyses are indicative of a dimeric protein in solution. Furthermore, we were able to confirm the formation of the conserved disulfide bridges, previously reported in fish STC1, by mass spectrometry.

## Results and Discussion

### STC1 is predicted to be dimer and to possess a high content of alpha-helices

By analysing the human STC1 amino acid sequence using six different secondary structure prediction databases, we created a secondary structure consensus and scored it by the number of times (one to six times) the predicted secondary structure element scored positive (Fig. 1). Prediction programs used were: **PredictProtein/PROF** [51]**, PsiPRED** [52]**, Predator** [53]**, SOPMA** [54]**, SSPro** [55] and **JCFO** [56].

In summary, the secondary structure analysis suggested that about 34% of the amino acid sequence of STC1 may form alpha-helices. Such a relatively high content is supposed to be readily detected by circular dichroism spectroscopy of the protein. As we will show further down, a high alpha-helical content was confirmed experimentally later on.

We further performed some predictions about ordered or disordered regions within the sequence using **FoldIndex** [57] and **DisEMBL** [58]) as well as **GlobPlot** [59] as a predictor for more globular regions (Fig.1). The first two programs both predicted that the pro-peptide region and possibly the C-terminal region, this last one which contains the Cys disulfide mediated dimerization region, to be highly disorder or a region with high loop/turn content.

We analyzed and plotted (Fig.1) the conserved cysteine residues as well as the experimentally determined disulfide bridges from the salmon sequence determination [39], the signal peptide, pro-peptide and mature protein sequence as annotated at

UniProtKB/Swiss-Prot database (Swiss-Prot:P52823), and we also emphasize a previously described nucleotide binding domain (NBD) [60].

Additionally, some predictions about post-translational modifications were performed and compared to published experimental data. A N-glycosylation site which had already been characterized for STC1 [17,41,61] was also predicted by **NetGlyc** [62] (Fig.1.)

For phosphorylation analysis we combined prediction data from **NetPhos** [63] and **NetPhosK** [64] together with *in vitro* phosphorylation data [50] to annotate tyrosine, threonine and serine residues as putative phosphorylation sites (Table 1). Most of the kinases that were found to phosphorylate STC1 by the in vitro phophorylation screening of Jellinek and coworkers were predicted by both prediction programs (Tab.1), except calmodulin-dependent protein kinase (CaMPK-II) and casein kinase II (CK2).

Analysis by **PredictProtein/PHD Acc** [51] revealed that some of the residues such as $S_{176}$ and $T_{216}$, are predicted to be exposed to the solvent and therefore more likely

to suffer phosphorylation. Indeed, both residues refer to STC1 kinase sites found to be phosphorylated by Jellinek and co-workers [50].

In order to screen for lysine residues predicted which may be sumoylated in STC1 we used SUMOplot™ **(**http://www.abgent.com/tools/sumoplot) and found three putative sumoylated residues (data not shown). The one having the highest score is located at the end of the NBD and the sumoylated residue ($K_{83}$) is also predicted by **PredictProtein/PHD Acc** to be exposed to solvent. Most interestingly, we found that STC1 interacted with the SUMO1 protein in a yeast two hybrid screen (unpublished data). These data suggest that further experiments should be performed to test if sumoylation of STC1 may occur in vivo, in human cells.

**Optimization of the expression and purification of STC1**

Our first attempt to produce STC1 in *E. coli* using the HT-STC1ΔNterm construct (Fig. 2A) resulted in completely insoluble expression (Fig. 2B). Even splitting the protein in two halves using His-tag fusion did not make any difference in solubility, since both parts still expressed in insoluble form (data not shown). Only together with the use of GST-tag (GST-C STC1) we could obtain some soluble expression, however at very low amounts. The highest rate of soluble expression could be obtained with GST-C STC1 (Fig. 2C and D).

On the other hand, using a modified bi-cistronic vector of the baculovirus expression system we could obtain milligrams per liter of the soluble full-length his-tagged STC1 (STC1-HT) secreted into the media (Trindade et al., unpublished data). The amount of virus and of infected cells could be easily optimized, since the recombinant bi-cistronic baculo virus promotes production of endogenous GFP protein, turning infected cells green.

Purification was obtained by a three step chromatography of the media: cation exchange followed by metal-affinity and size exclusion chromatographies (Fig. 2E). Several milligrams of protein were routinely obtained per liter of culture supernatant and the obtained protein was used for subsequent experiments.

**Confirmation of disulfide bonds by mass-spectrometry**

By analysing the recombinant human STC1-HT produced in the baculovirus system by ESI/Q-TOF analysis we were able to identify and assign the peptides that resulted from enzymatic digestion either with trypsin or chymotrypsin in the oxidized and/or reduced forms (Table 2, supplementary Figure S1). In brief, the data show the existence of peptides having mass compatible with the presence of the previously predicted disulfide bonds for the salmon STC1. In Table 2 the first column gives the disulfide bridge in question and the last four columns give respectively the expected and experimentally determined peptide masses. In conclusion, all disulfide bridges except for one could be directly demonstrated. Still $Cys_{45}$-$Cys_{59}$ could be evidenced indirectly, since the mass of the peptide shown in line one of Table 2 is compatible with this interpretation. Furthermore, after chemical digestion with formic acid, $Cys_{202}$ could be unambiguously assigned as the Cys residue responsible for the dimerization of human STC1 (Table 2, supplementary Figure S1).

**Analysis of secondary structure**

The content of secondary structure elements in recombinant human STC1-HT was determined by circular dichroism spectroscopy. Figure 3 shows the spectrum of purified STC1 recorded at 4 °C. Purified protein presents negative ellipticity in the near-UV, with minima at 208 (-17.2 $x10^3$ deg $cm^2dmol^{-1}$) and 222 nm (-12.8 $x10^3$ deg $cm^2dmol^{-1}$). Deconvolution of the CD spectrum lead to the following estimation of the content of secondary structural elements: ~52% of α-helices, ~19% of β-sheets
strands, ~11% of turns and ~18% unordered (NRMSD=0,009) using the CDSSTR algorithm on the Dichroweb web server [65]. Consensus predictions of secondary structures shown in Fig. 1 give values of about 37% of helix, 2.5% of strands and 65.5% of other structures (37% of coils and 28.5% of non-determined). Secondary structural predictors like PSIPRED are based on neural networks trained on known folds, and thus tend routinely to underestimation of the true helical and strand content, due to the fact that the reference databases are not complete. A more critical issue is the fact that no other protein of the family of STCs has its structure resolved. In conclusion both the prediction and the

experimentally determined data are in reasonable agreement, since they demonstrate a relatively high content of alpha-helices in human STC1.

**STC1 is a compact, slightly ellipsoidal dimer in solution**

Dynamic Light Scattering (DLS) data of the recombinant STC1 sample showed a single and narrow peak, a predicted mass and a percentage of poly-dispersity indicative of a monodisperse solution of dimers.

The corrected and normalized experimental SAXS data are shown in Figure 4A, together with the GNOM curve fitting. The Guinier region providing an Rg value of 27.4 ± 0.8 Å is shown in the inset. The p(r) function resulting from these calculations is shown in Fig. 4B, with an inset showing the Kratky representation of the intensity curve. The Kratky plot indicates a slightly compact conformation for STC1 in solution. The maximum dimension ($D_{max}$) value obtained was 90 Å and the Rg value, calculated from the p(r) function, was 27.8 ±0.4 Å, in close agreement with that calculated from the Guinier approximation. As it can be noted from the p(r) function shape, STC1 has a slightly elongated shape.

Using BSA as a reference sample, the molecular mass for STC1 HT, estimated from the SAXS data, was ~ 54 kDa. This value is in agreement with the prediction of the protein being a dimer, since the theoretically calculated molecular mass of the monomer was ~27 kDa (calculated from the aminoacids sequence using ProtParam tool [66]).

The dimerization was also confirmed both by mass spectrometry (see above) and by size exclusion chromatography (data not shown).

**Low resolution *ab initio* SAXS-based models for STC1**

The low resolution models for STC1 are presented in figure 5. Those models were derived from the experimental SAXS data imposing a 2 point symmetry constraint (P2). Additional models calculated without symmetry constraint (P1) presented very similar molecular envelopes. The calculated values of the Normalized Spatial Discrepancy (NSD), which is indicative of the difference between models, gave values ~0.6 for P1 vs. P2 DAMMIN models and ~0.8 for P1 vs. P2 GASBOR models, indicating a low discrepancy. In view of this result, all model calculations were performed using a 2 point symmetry constraint. After several runs performed with the program DAMMIN, the averaged and filtered (with the corrected excluded volume) *dummy atom* model for STC1 is shown in figure 5(A). The NSD values for the set of 10 models ranged from 0.60 to 0.69, which are considered reasonable values [67]. This low resolution model shows the expected elongated shape for the protein dimer. The most typical and recurrent *dummy residue* model resulting from the calculation

with the program GASBOR is shown in figure 5(B). The NSD values for this set of 10 calculations ranged from 0.82 to 0.87, which are also quite reasonable. This last approach produced an improved molecular envelope for STC1. Comparing the results, both molecular envelopes obtained for STC1 presented a similar shape and confirmed the elongated conformation for the dimer.

# Conclusions

Our data provided the first low resolution 3D structure of human STC1 protein in solution. SAXS experiments indicated that STC1 forms a dimer of slightly elongated shape in solution. Circular dichroism spectroscopy confirmed the prediction of a high alpha-helical content and we could also confirm by mass spectrometry the highly conserved disulfide pattern, previously described in fish STC1[39]. Disulfide bonds are formed between the same 10 of the 11 conserved Cys, in the same fashion, leaving the C-terminal Cys 202 free to engage in dimer formation. None of our data explain the composition or structure of "bigSTC1" previously reported to appear in certain tissues [19,25,46-49] . Indeed, our results only show the formation of dimers ($STC_{50}$), by several independent methods. In human cells however, we may have additional contributions from possible post-translational modifications or alternative splice variants of the pre-mRNA encoding STC1, which may contribute to the appearance of the higher molecular weight forms. Further experiments are required to characterize big STC1 at the molecular level and point out its differences with the canonical dimeric human STC1.

# Methods

### *In silico* sequence analysis

We analyzed the human STC1 sequence as a query in six different secondary structure prediction databases (PredictProtein/PROF [51], PsiPRED [52], Predator [53], SOPMA [54], SSPro [55] and JCFO [56]). We also performed some predictions about ordered or disordered regions within the sequence using FoldIndex [57] and DisEMBL [58]) as well as GlobPlot [59], a predictor for globular regions.

Additionally, some predictions about post-translational modifications were done and compared to previous published data. N-glycosylation sites were predicted by NetGlyc [62]. For phosphorylation we combined prediction data from NetPhos [63] and NetPhosK [64]. With PredictProtein/PHD Acc [51], we predicted whether residues are exposed to solvent or buried. Finally, in order to screen for lysine residues predicted which may be sumoylated in STC1 we used SUMOplot™ (http://www.abgent.com/tools/sumoplot).

**Cloning of STC1 cDNA**

Full-length STC1 (Genbank NM_003155) gene was amplified from normal bone marrow stromal cells using primers STC1 F (5' aaggatccAGAATGCTCCAAAACTCAGC 3') and STC1 R (5' ccgaattCCTCTCCCTGGTTATGCAC 3') and cloned into vector pGEM resulting in plasmid **pGEM-STC1**. In order to obtain all constructs we used pGEM-STC1 as template and cloned PCR amplified products into pGEM plasmid: for **pGEM-STC1 $\Delta N_{term}$** ($\Delta N_{term}$ lacking the first 22 aminoacids) we used primers STC1 $\Delta N_{term}$ F (5' aaggatccCAGAATGACTCTGTGAGCCC 3') and STC1 R; for **pGEM-STC1 full without stop** (STC1 without stop-codon) we used primers STC1 F and STC1 no stop R (5' acaagcttCCTCTCCCTGGTaATGCAC 3'); for **pGEM-CSTC1** (C terminal of STC1 consisting of residues from 129 to 247) were used primers CSTC1 F (5' ggatccTACAGCAAGCTGAATGTGTG 3') and CSTC1 R (5' gaattcTTATGCACTCTCATGGGATG 3'). Capital letters indicate sequence identical to STC1 cDNA, small caps letters indicate sequence non-identical to template. All pGEM constructs were verified by DNA sequencing in order to ascertain the correct nucleotide sequence. pGEM-STC1 $\Delta N_{term}$ and pGEM-CSTC1 were digested with BamHI and EcoRI and the resulting inserts were cloned into pET28a-His-Tev or pET28a-GST-Tev [68] previously digested with the same endonucleases. This resulted in pET-HT-STC1 $\Delta N_{term}$, pET-HT-CSTC1, and the pET-GST-CSTC1 constructs. pGEM-STC1 full without stop was digested with BamHI and HindIII and cloned into a pFastBAC Dual+EGFP (pFBDg), which had the EGFP cDNA cloned under p10 promoter, digested with same endonucleases to insert STC1 under polyhedron promoter. Subsequently a pair of oligos (5'AGCTTGGAAAACCTGTATTTTCAGGGCCATCACCATCACCATCACCGG 3' and 5'AGCTCCGGTGATGGTGATGGTGATCGCCCTGAAAATACAGGTTTTCCA 3') previously annealed was added to generate a linker consisting of a TEV protease site and a 6xHis-tag (HT) at the C-terminal, resulting in the pFBDg-STC1-HT construct. Other constructs mentioned in the text were generated by using the same methodology.

**Expression and purification of STC1**

Production of the recombinant 6xHis- or GST-STC1 fusion constructs in *E. coli* BL21 strain and subsequent purification trials were performed as described previously for other recombinant proteins [69,70].

High Five™ (Invitrogen) cells were adapted to grow in suspension culture in Express Five™ serum free media (Gibco) supplemented with 20mM L-Glutamine (Gibco) and 1x PenStrep

(Gibco). The stock cell culture was maintained and passaged in a 28 °C incubator (ThermoForma). For STC1-HT production High Five cells were scaled up from the stock culture to a cell density of $1x10^6$ in two 2L Erlenmeyer flasks containing 500 mL each and incubated at a shaker at 26 °C at 140 rpm. Twelve hours post inoculation, the cells were infected with the recombinant baculo virus, at a multiplicity of infection (m.o.i.) between 3 and 4 plaque-forming unit (pfu) per cell.

Baculovirus-infected High Five culture media were harvested after 48 hours post-infection by centrifugation at 500 x g for five minutes and cell-free supernatant containing secreted STC1-HT was used for purification. To the baculovirus supernatant a 1M MES stock solution was added to bring the solution to a final concentration of 50 mM MES pH 6.5 (IEX buffer)

The solution was filtered through a 0.45 µm MCE membrane (Fisherbrand) and loaded onto a water-jacketed chilled (4 °C) XK26/20 (Pharmacia Biotech/GE) column previously packed with SP Sepharose FF (Pharmacia Biotech/GE) at a flow rate of 1mL/min using a peristaltic pump (Biologic LP – Biorad). Column was transferred to a ÄKTA FPLC system (GE) for protein elution using a 0-1 M gradient of NaCl in IEX buffer. Fractions eluted from a conductivity of 30 mS/cm onward, contained most of stanniocalcin 1 protein and were pooled. This pool was directly loaded onto a pre-packed HisTrap crude FF 5 mL (GE) column, equilibrated with 50 mM MES pH 6.5, 500 mM NaCl (affinity buffer). After injection of sample the column was washed with six column volume (CV) of affinity buffer, with three CV of affinity buffer containing 250 mM Imidazole and finally with four CV of affinity buffer containing 1M Imidazole. This last pool of fractions containing most of stanniocalcin was concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane of 5,000 NMWL (Millipore) on a swing-rotor at 4 °C and then 500 µL applied to a water-jacketed chilled (4 °C) Superdex 200pg 16/60 (GE) column, pre-equilibrated with 60 mM MES 600 mM NaCl pH 6.5 (SizeEx buffer) with a flow rate of 0.5 mL/min. Protein eluted at a single peak between 70 and 80 mL, was analyzed by SDS-PAGE, pooled, concentrated and kept in SizeEx buffer at 4 °C.

**Disulfide bond and molecular mass analysis**

Samples digested by trypsin or chymotrypsin, treated or not with dithiotreitol and iodoacetamide, were analyzed by using ultra-performance liquid chromatography (UPLC NanoAcquity, Waters) coupled with eletrospray ionization quadrupole time-of-flight tandem mass spectrometer (ESI-QTOF Ultima, Waters/Micromass). Samples chemically digested by formic acid [71], treated or not with dithiotreitol and iodoacetamide, were analyzed using

MALDI-QTOF (Q-Tof Premier, Waters/Micromass). Data were analyzed by the MassLynx software package.

**Circular Dichroism**

Circular dichroism spectra were recorded at 4 °C between 190 and 260nm on a J-810 Jasco spectropolarimeter equipped with a Peltier-type system PFD 425S using a quartz cuvette of 10mm path length, with a 50 nm/min scanning speed and a band-width of 0.5 nm. Twenty spectra of purified STC1-HT at 2.77 μM in dilution buffer (10mM MES 33.3 mM NaCl pH6.5) were averaged and corrected from the baseline for buffer solvent contribution. Experimental data were analyzed using CDSSTR on Dychroweb web server [65].

**Small Angle X-Ray Scattering and Analysis**

Before the analysis, the sample was inspected by dynamic light scattering (DLS) to test the monodispersity of the solution. After that, the sample was centrifuged at 20.000×g for 30 min at 4 °C to remove any possible aggregates. The small-angle X-ray scattering experiments were performed at the D02A-SAXS2 beam line at LNLS. The measurements were performed at 4 °C under temperature-controlled conditions (via water circulation) using a 1 mm path length cell with mica windows and a monochromatic X-ray beam (wavelength of $\lambda$ = 1.488 Å). The X-ray patterns were recorded using a two-dimensional position-sensitive MARCCD detector and a sample-to-detector distance of 902 mm, resulting in a useful scattering vector range of $0.015 Å^{-1} < q < 0.25 Å^{-1}$, where q is the magnitude of the **q**-vector defined by $q=(4\pi/\lambda)\sin\theta$ ($2\theta$ is the scattering angle). Three successive frames of 300 seconds each and one frame of 30 minutes were recorded. The measurements were performed with two different concentrations for the sample in MES buffer (60mM MES 200mM NaCl pH 6.5): 0.15 and 0.18 mg/mL, both measured using the BCA™ Protein Assay Kit (Pierce). The buffer scattering curves were recorded keeping the same conditions used for the sample. The intensity curves were individually corrected for detector response and scaled by the incident beam intensity and sample absorption. Subsequently, buffer scattering was subtracted from the corresponding sample scattering. The resulting curves were inspected for radiation-induced damage, but no such effect was observed. After scaling the curves for concentration, no concentration effect was observed. A 10 mg/ml BSA (66 kDa) solution in the same sample buffer was used as molecular mass standard sample to estimated the molecular mass of STC1-HT. This value was inferred from the ratio of the extrapolated values of the intensity at the origin, I(0), from both sample and BSA solutions scattering [72,73].

The first analysis was the evaluation of the radius of gyration (Rg) using the Guinier approximation: $I(q) = I(0)\exp(-q^2Rg^2/3)$ for $qRg$ < 1 [74-76]. The Rg was also calculated from the pair distance distribution function, p(r), which was obtained by indirect Fourier transform of the intensity curve using the program GNOM [77]. The p(r) function also provided the maximum dimension ($D_{max}$) of the molecule, Moreover, a Kratky representation [75,76] of the intensity curve ($q^2I(q)$ vs. $q$) was used to analyze the compactness of the protein conformation.

***Ab initio* SAXS-based modeling**

The low resolution models for STC1 were restored from the SAXS intensity curves using two different approaches. In the first one, implemented by the program DAMMIN [78], the protein was represented as an assembly of densely packed spherical beads (*dummy atoms*). Using simulated annealing, the program starts from a random configuration of beads and searches for a configuration that best fits the experimental pattern. Ten calculations were performed and normalized spatial discrepancies (NSD) [67] values among them were evaluated using the DAMAVER suite. When the NSD values are not so different, an averaged and filtered model structure (with the correct excluded volume) emerges from this calculation. The second approach, in which generally a better model is obtained, was implemented using the program GASBOR [79]. In this approach, the protein is represented as a chain of *dummy residues* (DRs). The number of DRs is usually known *a priori* from the protein amino acid sequence. Starting from a randomly distributed gas of DRs inside a spherical volume of diameter $D_{max}$, a simulated annealing routine was employed to find a chain-compatible spatial distribution of DRs which would fit the experimental scattering pattern. Ten different calculations were also performed and the NSD values were evaluated. In this case, there is no advantage in obtaining an average model because the GASBOR program uses a predefined number of DRs, which makes the average routine little effective in achieving an improvement of the model resolution. So, we present the most typical model (with the lowest NSD value). In both approaches, the models calculated with 2 point-symmetry constraint were very similar to those calculated without these constraints. For this reason, the results presented here are from the calculation with 2 point-symmetry constraint.

## Authors' contributions

DMT and JK conceived and designed the experiments, analyzed the data and wrote the manuscript. DMT performed the experiments. JCS performed SAXS experiments and interpreted them together with ICLT. MSN performed and interpreted the mass spectrometry experiments. JK supervised the project.

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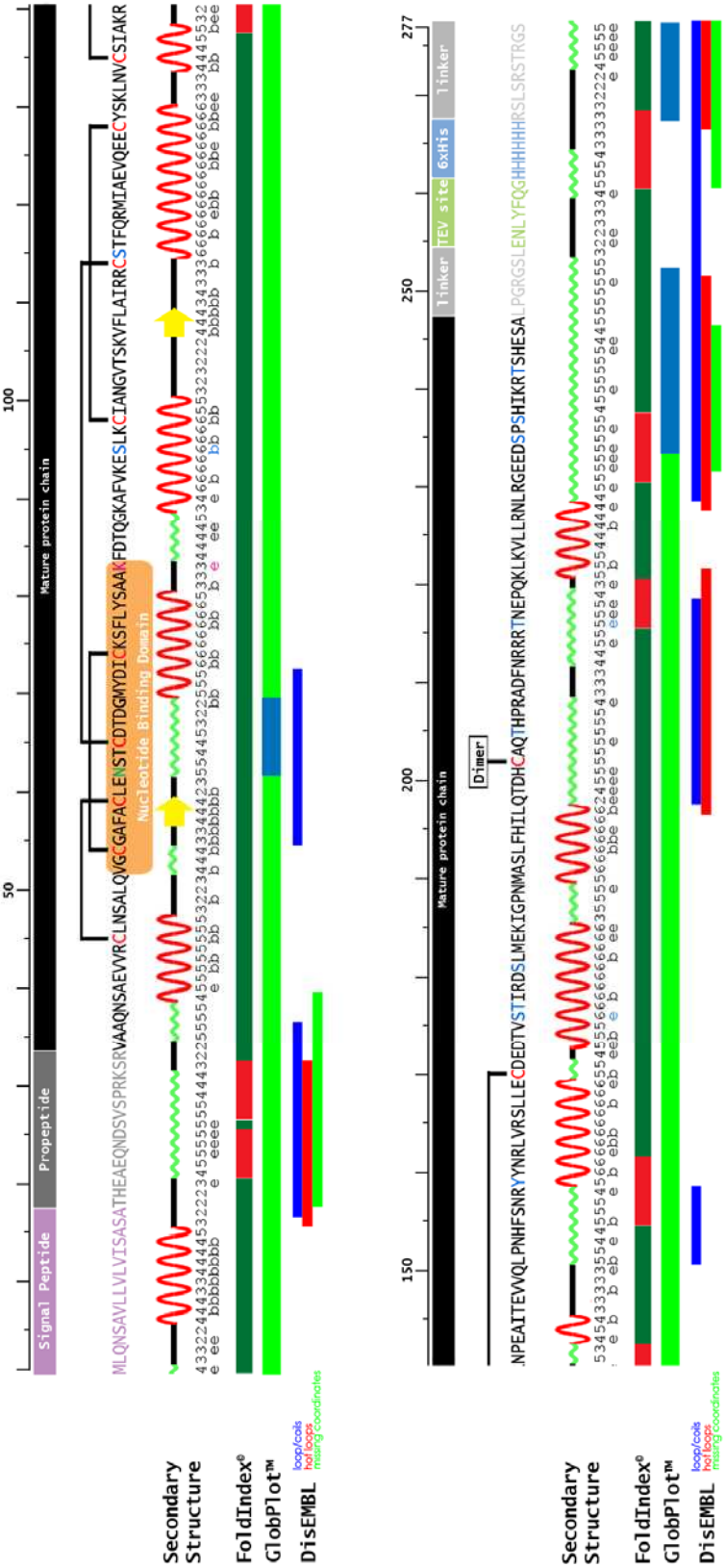
# Figures




*(Legend next page →)*

*(← previous page)* **Figure 1 - Prediction of secondary structure and putative post-translational modification sites in the human STC1 amino acid sequence.**
Linear representation of STC1-HT amino acid sequence with assignment of its different regions from N- to C-terminus: signal peptide (purple), pro-peptide (dark gray), mature protein (black), linker regions (light grey), TEV protease cleavage site (green) and 6xHis-tag (light blue). In the amino acid sequence relevant residues are emphasized by the following color code: Cys: red, Asp predicted to be N-glycosylated: green, Lys predicted to be sumoylated: magenta, Ser, Thr e Tyr residues predicted to be phosphorylated: blue. The conserved pattern of experimentally determined disulfide bridges from salmon STC1 is indicated by black horizontal brackets. Similarly the homo-dimerization Cys is indicated in black (dimer). Below the sequence there is a schematic representation of the predicted consensus secondary structure, obtained by six different prediction programs (red: alpha helix, yellow: beta-sheet, green: coil regions, black: not assigned). The numbers below the secondary structure represent the score (1-6, indicating how many of the six programs predicted the respective secondary structure element). Furthermore, in a second line, a prediction indicates whether a residue is exposed (e) or buried (b). At the bottom, predictions of three programs for ordered/disordered regions are given: FoldIndex (red: unfolded, green: folded), GlobPlot (green: globular, blue: disordered) and DisEMBL (blue: loops or coils, red: hot loops, green: missing coordinates).

*(next page →)* **Figure 2 - Large scale STC1 expression in E. coli and in insect cells and its purification.**
**(A)** Tested STC constructs (from top to bottom): an amino-6xHis tagged STC1 without the N-terminal portion which includes the signal peptide (HT-STC1ΔNterm), an amino-GST tagged C-terminal fragment of STC1 (GST-C STC1) and a full length carboxy-6xHis tagged STC1 (STC1-HT). At the right side of each construct is shown the aminoacid residues from native STC1 present on that construct. **(B)** Expression test of HT-STC1ΔNterm. Comassie-blue stained SDS-PAGE of soluble (S) and insoluble (I) fractions expressed in BL21DE3 non-induced (NI) or induced for indicated periods with 0.5 mM IPTG in LB at 37 °C. **(C)** GST-C STC1 purification by affinity chromatography using glutathione sepharose beads. Comassie-blue stained SDS-PAGE of insoluble (I), soluble (S), flow-through (FT), wash (W) and elution (E1-E3) fractions. **(D)** Western blot anti-GST of E2 fraction of purification shown in C. Black arrow heads at right indicate expected recombinant protein size and red arrow head indicates un-fused GST protein. **(E)** Expression and purification of STC1-HT from insect cells (using the baculo virus system): comassie-blue stained SDS-PAGE of peak-fractions after cation exchange, metal affinity and Size Exclusion chromatography. Arrow head indicates expected size of recombinant expressed protein. Invitrogen Bench Marker protein ladder (M).

*(← Legend prev. page)*

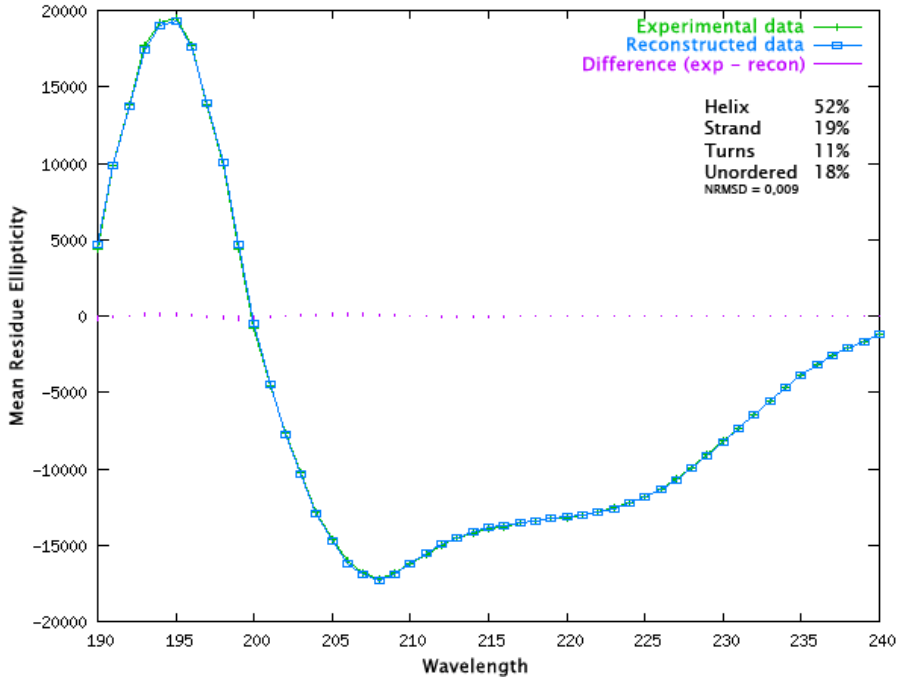



**Figure 3 - Circular Dichroism spectra of STC1-HT and deconvolution**
Graph of the wavelength plotted against the mean residue elipticity of a sample at 5,5 mM in 10 mM MES; 33,3 mM NaCl pH 6,5 at 4 °C. Data were deconvoluted with the CDSSTR program on the Dichroweb server. Note the two minima at 208 and 222 nm, which are typical of alpha-helix containing proteins. Reconstructed data are those derived from the Dichroweb database.

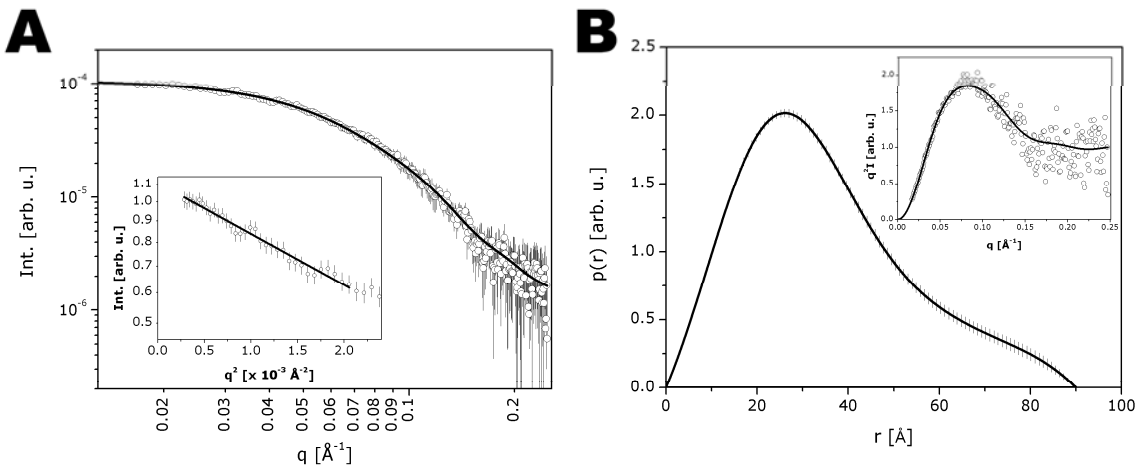



**Figure 4 - Experimental Small Angle X-ray Scattering (SAXS) curves for recombinant STC1-HT protein.**
(A) Experimental scattering curve of STC1-HT (open circles) and the theoretical fitting (solid line) by using the program GNOM. *Inset*: Guinier Region. (B) Pair distance distribution function p(r). *Inset*: Kratky representation of the intensity curve.

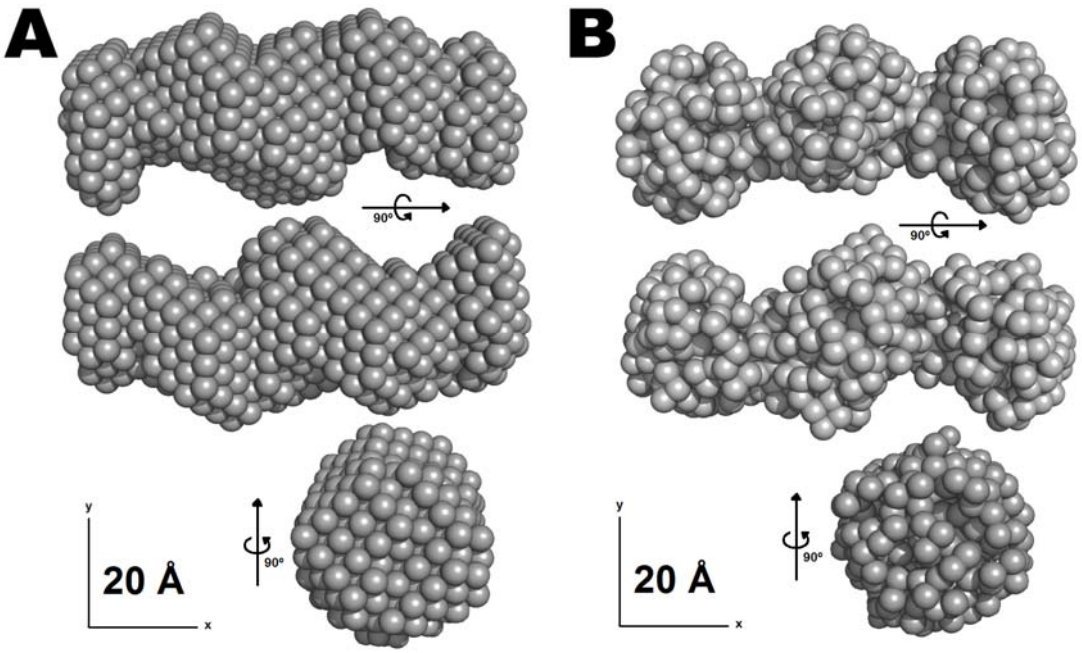



**Figure 5 - Low resolution *ab initio* model of STC1-HT derived from SAXS data.**
(A) Three selected views of the average and filtered *dummy atoms* model (DAMMIN). (B) Three selected views of the *dummy residues* model (GASBOR). The models were displayed by the PyMOL program [80].

# Tables

**Table 1 - Prediction of putative post-translational modification sites in human STC1.** High score predictions of glycosylation, sumoylation and phosphorylation on STC1 sequence are presented. Predicted modifications within the pro-peptide region were excluded. Putative phosphorylated residues shown here are only those that were both predicted with the highest scores by the NetPhos server and additionally were predicted by NetPhosK, which suggests a specific kinase for the same site. References are related to additional experimental support for the predicted modification, if available.

| Residue | Modification | Burried/ Exposed Residue[51] | Predictor (Score) | Ref. |
|---|---|---|---|---|
| $N_{62}$ | N-glycosylation | nd | NetGlyc (0.61) | [17,39, 41,61] |
| $K_{83}$ | Sumoylation | E | SUMOplot™ (0.79) | $ |
| $S_{95}$ | PKC* phosphorylation | B | NetPhos (0.844) / NetPhosK (0.630) | [50] |
| $S_{115}$ | PKC* phosphorylation | nd | NetPhos (0.788) / NetPhosK (0.722) | [50] |
| | PKA* phosphorylation | nd | NetPhos (0.788) / NetPhosK (0.841) | [50] |
| | RSK* phosphorylation | nd | NetPhos (0.788) / NetPhosK (0.601) | Nd |
| $Y_{159}$ | INSR* phosphorylation | nd | NetPhos (0.929) / NetPhosK (0.539) | Nd |
| $S_{176}$ | PKC* phosphorylation | E | NetPhos (0.938) / NetPhosK (0.630) | [50] |
| $T_{177}$ | PKC* phosphorylation | nd | NetPhos (0.983) / NetPhosK (0.640) | [50] |
| $S_{181}$ | PKA* phosphorylation | nd | NetPhos (0.993) / NetPhosK (0.647) | [50] |
| $T_{205}$ | PKC* phosphorylation | nd | NetPhos (0.606) / NetPhosK (0.815) | [50] |
| | Cdc2* phosphorylation | nd | NetPhos (0.606) / NetPhosK (0.509) | [50] |
| $T_{216}$ | PKG* phosphorylation | E | NetPhos (0.817) / NetPhosK (0.600) | [50] |
| $S_{235}$ | GSK3* phosphorylation | nd | NetPhos (0.986) / NetPhosK (0.508) | [50] |
| | Cdk5* phosphorylation | nd | NetPhos (0.986) / NetPhosK (0.551) | [50] |
| $S_{237}$ | PKC* phosphorylation | nd | NetPhos (0.531) / NetPhosK (0.647) | [50] |
| $T_{242}$ | PKG* phosphorylation | nd | NetPhos (0.523) / NetPhosK (0.693) | [50] |

*as predicted by NetPhosK; nd = not determined; $ = unpublished data; protein kinase A C or G (PKA; PKC and PKG); 90-kDa Ribosomal S6 Kinase ($pp90^{RSK}$ or RSK); Insulin receptor (INSR); cell division cycle 2 (cdc2 or p34 protein kinase); ciclin dependent kinase 5 (cdk5); Glycogen synthase kinase 3 (GSK3).

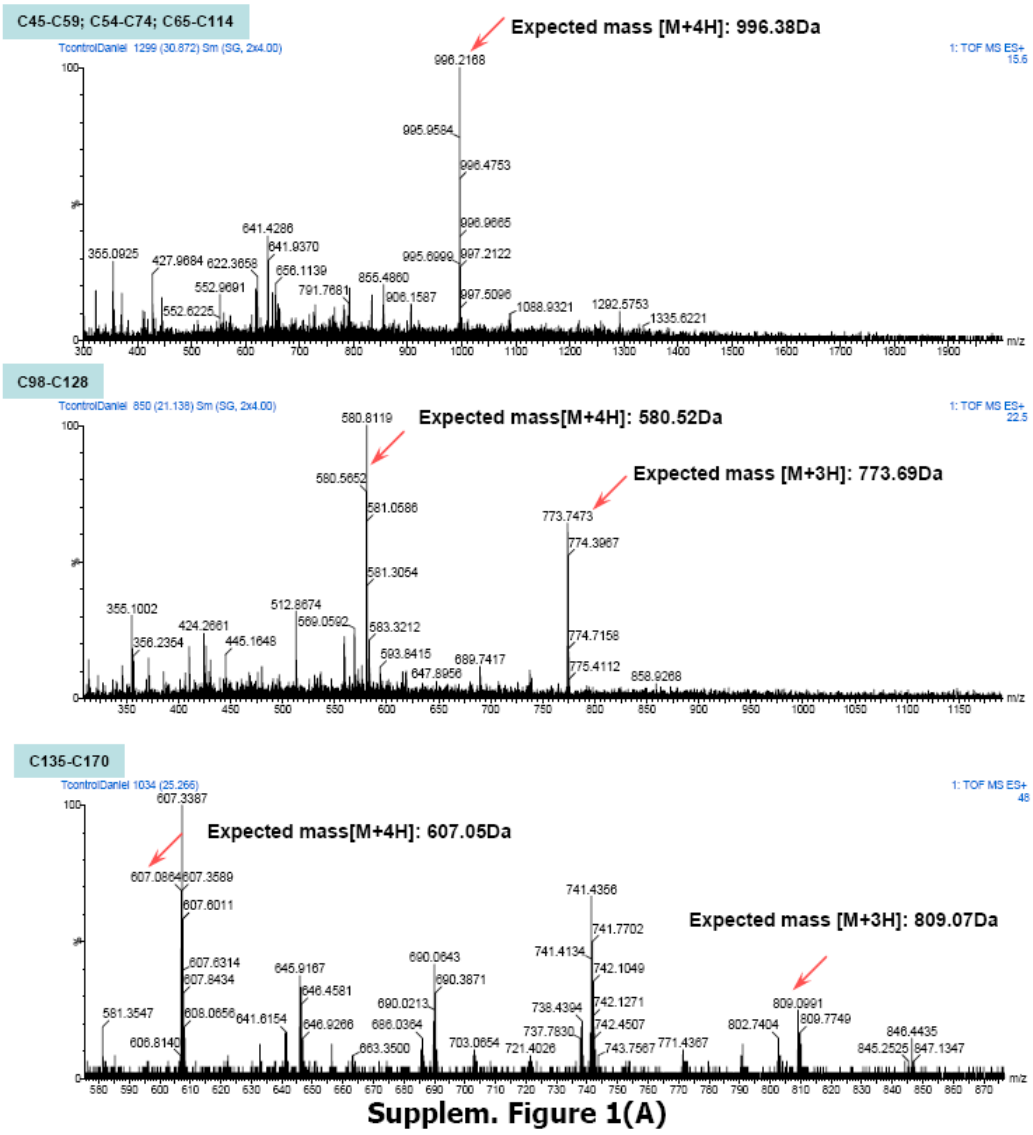
**Table 2 - Identification of signature peptide sequences of STC1-HT for the assignment of the intra- and intermolecular disulfide bonds.** Samples were digested by trypsin or chymotrypsin with or without dithiotreitol and iodoacetamide, separated by UPLC and analyzed by ESI-QTOF; or digested with formic acid and analyzed by MALDI-QTOF. The presented mass is the monoisotopic. Dimer disulphide bond is indicated by asterisk (*). (See spectra in supplementary figure S1).

| Disulfide Bond | Sequence of peptides | Protease | Mass | | | |
|---|---|---|---|---|---|---|
| | | | Theoretical | Observed (Expected) | | |
| | | | | [M+2H] | [M+3H] | [M+4H] |
| $C_{45}$-$C_{59}$; $C_{54}$-$C_{74}$; $C_{65}$-$C_{114}$ | **C**$_{45}$LNSAL…ID**C**K$_{75}$ **C**$_{114}$STFQR$_{119}$ | Trypsin | 3981.51 | | | 996.22 (996.38) |
| $C_{54}$-$C_{74}$ | Q$_{51}$VG**C**GAFA$_{57}$ D$_{72}$I**C**KSF$_{77}$ | Chymotrypsin | 1389.61 | 695.95 (695.81) | 464.24 (464.21) | |
| $C_{65}$-$C_{114}$ | E$_{61}$NST**C**…GMY$_{71}$ A$_{110}$IRR**C**STF$_{117}$ | Chymotrypsin | 2184.89 | | 729.36 (729.30) | 547.26 (547.23) |
| $C_{98}$-$C_{128}$ | **C**$_{98}$IANGVTSK$_{106}$ M$_{120}$IAE…**C**YSK$_{131}$ | Trypsin | 2318.06 | | 773.73 (773.69) | 580.56 (580.52) |
| $C_{98}$-$C_{128}$ | K$_{97}$**C**IA…SKVF$_{108}$ Q$_{118}$RMIA…EE**C**Y$_{129}$ | Chymotrypsin | 2761.32 | | 921.17 (921.45) | 691.14 (691.34) |
| $C_{135}$-$C_{170}$ | L$_{132}$NV**C**SIAK$_{139}$ S$_{166}$LLE**C**…TIR$_{179}$ | Trypsin | 2424.19 | | 809.12 (809.07) | 607.08 (607.05) |

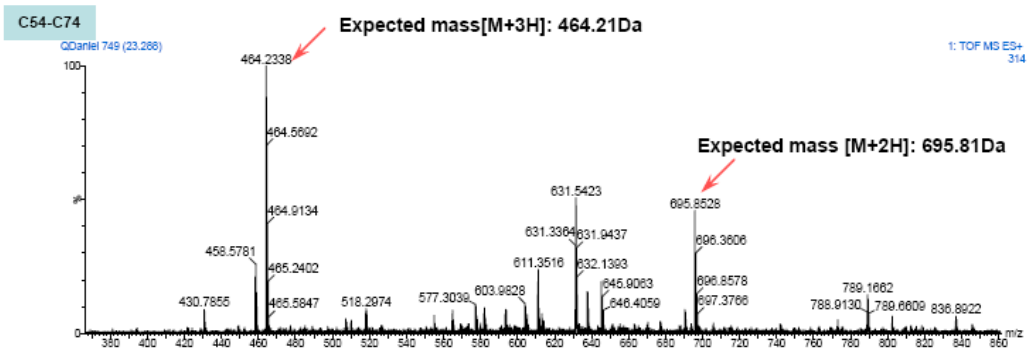
| Disulfide Bond | Sequence of peptides | Chemical Reagent | Mass | | |
|---|---|---|---|---|---|
| | | | Theoretical | Observed (Expected) | |
| | | | | [M+H] | |
| $C_{202}$-$C_{202}$ * | D$_{200}$H**C**AQTHPRA$_{209}$ D$_{200}$H**C**AQTHPRA$_{209}$ | Formic acid | 2266.98 | 2268.11 (2267.99) | |

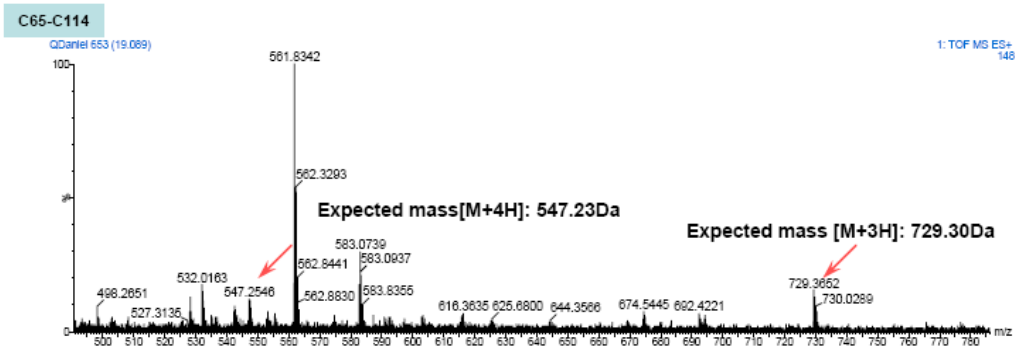
# Additional files

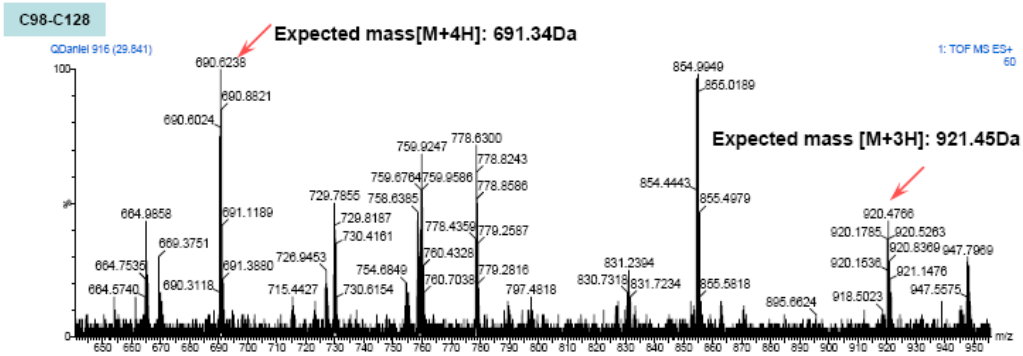
**Supplementary figure 1 – Original UPLC-ESI-QTOF and MALDI-QTOF data of the data presented in Table 2.**



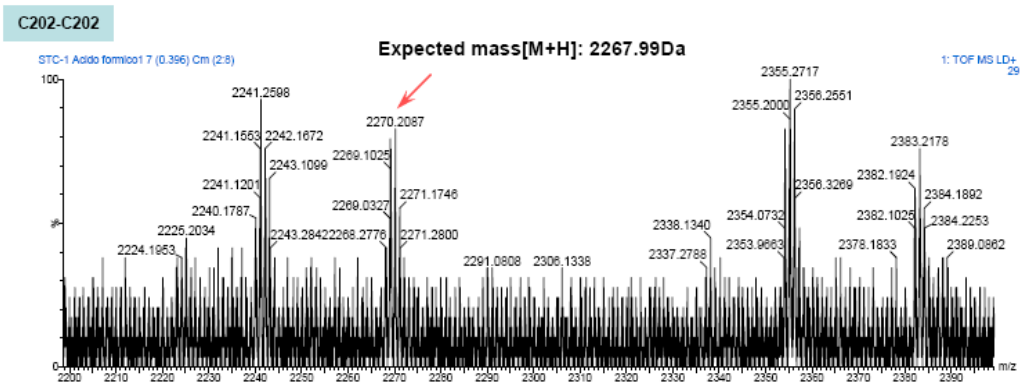

**Supplem. Figure 1(A)**



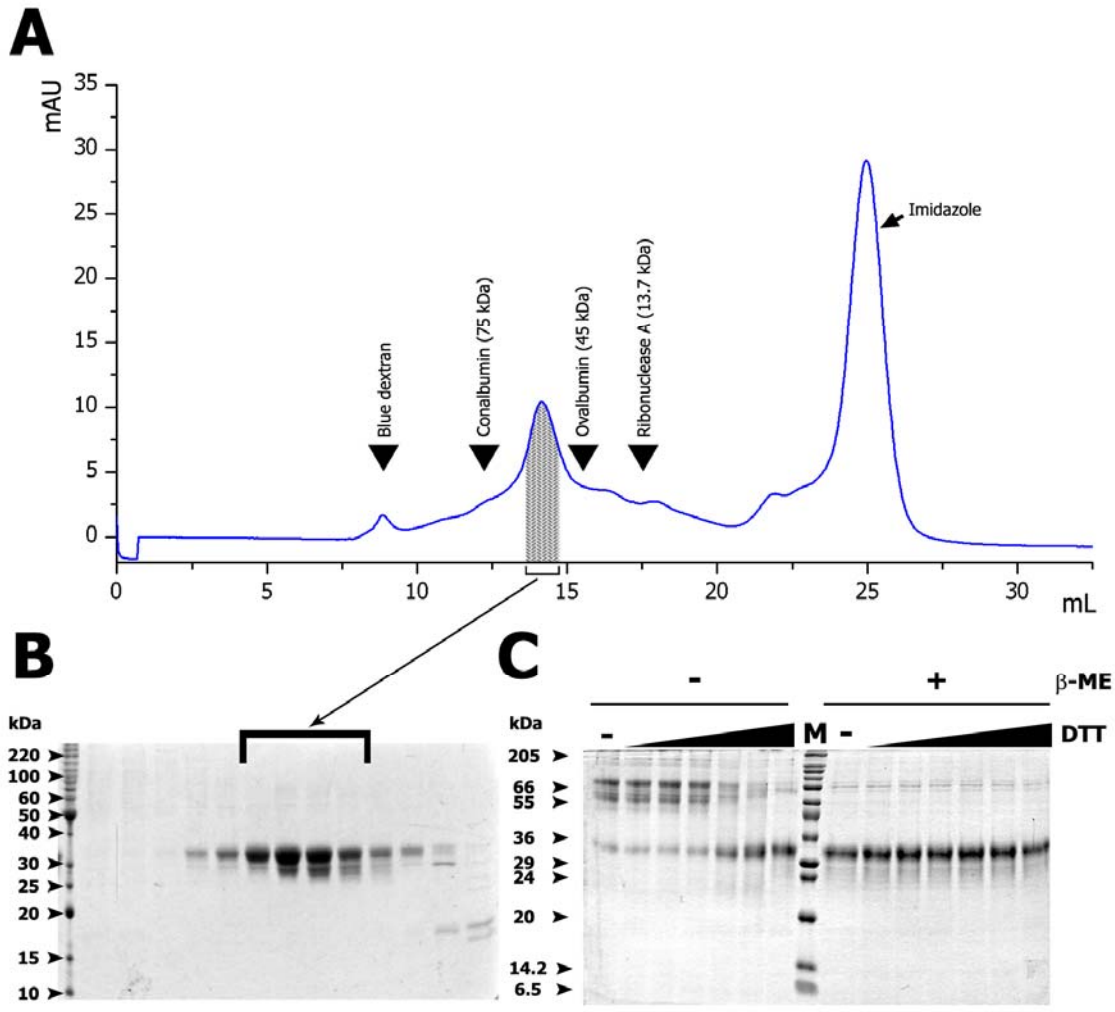




**Supplem. Figure 1(B)**




**Supplem. Figure 1(C)**




**Supplementary figure 2 – Size Exclusion and semi-native SDSPAGE confirm dimer.**
(A) UV profile of STC1-HT of semi-analitical size exclusion chromatography. Arrow heads indicate marker proteins and bluedextran (void). Due the high concentration of imidazole in the sample its possible to see a peak at the end of the run. (B) Comassie blue stainned SDS-PAGE of some samples of the semi-analitical size exclusion chromatography. Samples related to the STC1-HT peak are shown under brackets. (C) semi-native SDS-PAGE of STC1-HT with growing concentrations of DTT (0, 10, 50, 100, 500, 1000 and 5000 nM) prepared adding or not the beta-mercaptoetanol (β-ME) in the sample buffer.

# C. RESULTADOS COMPLEMENTARES

## 1. *Modificação do sistema de expressão de baculovírus*

A determinação do título viral no sistema de baculovirus normalmente é um processo um tanto quanto longo e árduo em grande parte devido ao ensaio de placa (*plaque assay*) o qual demanda grande habilidade por parte do pesquisador para a sua realização. Visando simplificar o processo de determinação do titulo viral para baculovirus recombinantes, construímos, utilizando os vetores pEGFP (Clontech) e pFastBac Dual (Invitrogen), um novo vetor, o pGreenBac (Figura 2) de modo à proteína fluorescente verde (GFP) funcionar como repórter de infecção. Deste modo tanto as colônias (no ensaio de placa para titulação) como as células infectadas são facilmente detectadas devido à expressão da EGFP, em transiluminador UV ou microscópio de fluorescência invertido (Figura 3).

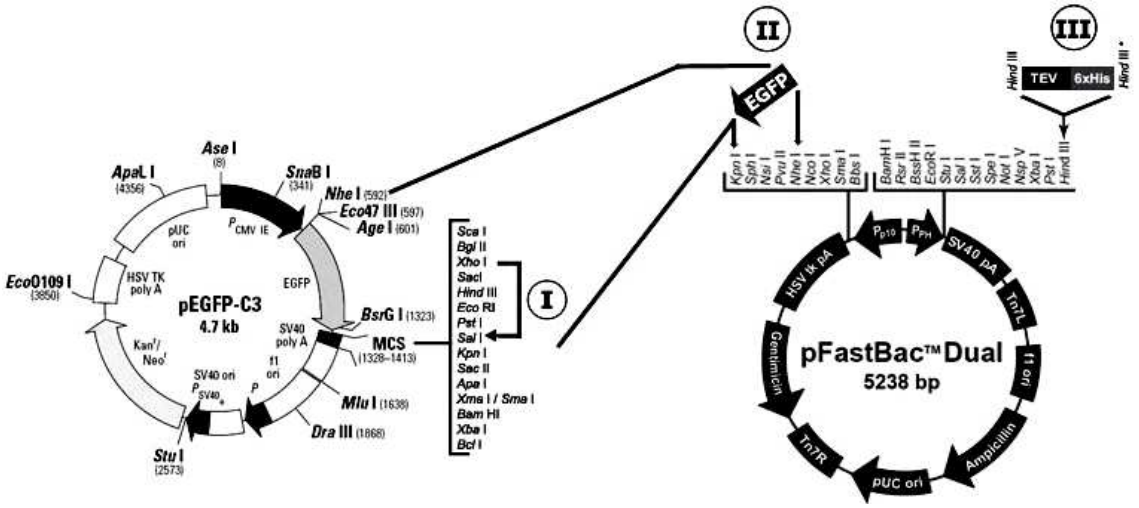



**Figura 2. Mapa da criação do vetor pFastBacEGFP C-HT.** Mapa mostrando a subclonagem da seqüência codificadora da versão mais brilhante da proteína verde fluorescente (EGFP), após retirada parte do *polylinker* do vetor pEGFP-C3 **(I)**, no *polylinker* do vetor pFastBac™Dual sob controle do promotor do gene p10 ($P_{p10}$) **(II)**, e da inserção do linker contendo o sitio de TEV e o 6xHis tag no sitio de *Hind* III do *polylinker* do mesmo vetor sob controle do gene da poli hedrina ($P_{PH}$) **(III)**.

Além disso, com base em uma estratégia muito semelhante à de Philipps e colaboradores (2005) otimizamos também o tempo de produção de um estoque de alto título de baculovirus. Normalmente os vírus são coletados entre três a quatro dias após a transfecção sem que as células sejam alimentadas ou subcultivadas e são necessárias outras duas ou três infecções a fim de produzir um sobrenadante com alto título viral. Uma vez que durante a transfecção inicial apenas cerca de 10% das células são transfectadas, pelo menos 90% das células ainda são susceptíveis de infecção e a principio podem dividir-se, contudo isso não ocorre devido à alta confluência. Contudo, fazendo o subcultivo das células, após dois

dias de transfecção, para uma área maior e acrescentando meio de cultura novo as células infectadas continuam a produzir vírus os quais infectam as demais células aumentando o título produzido nesses três ou quatro dias de infecção inicial.

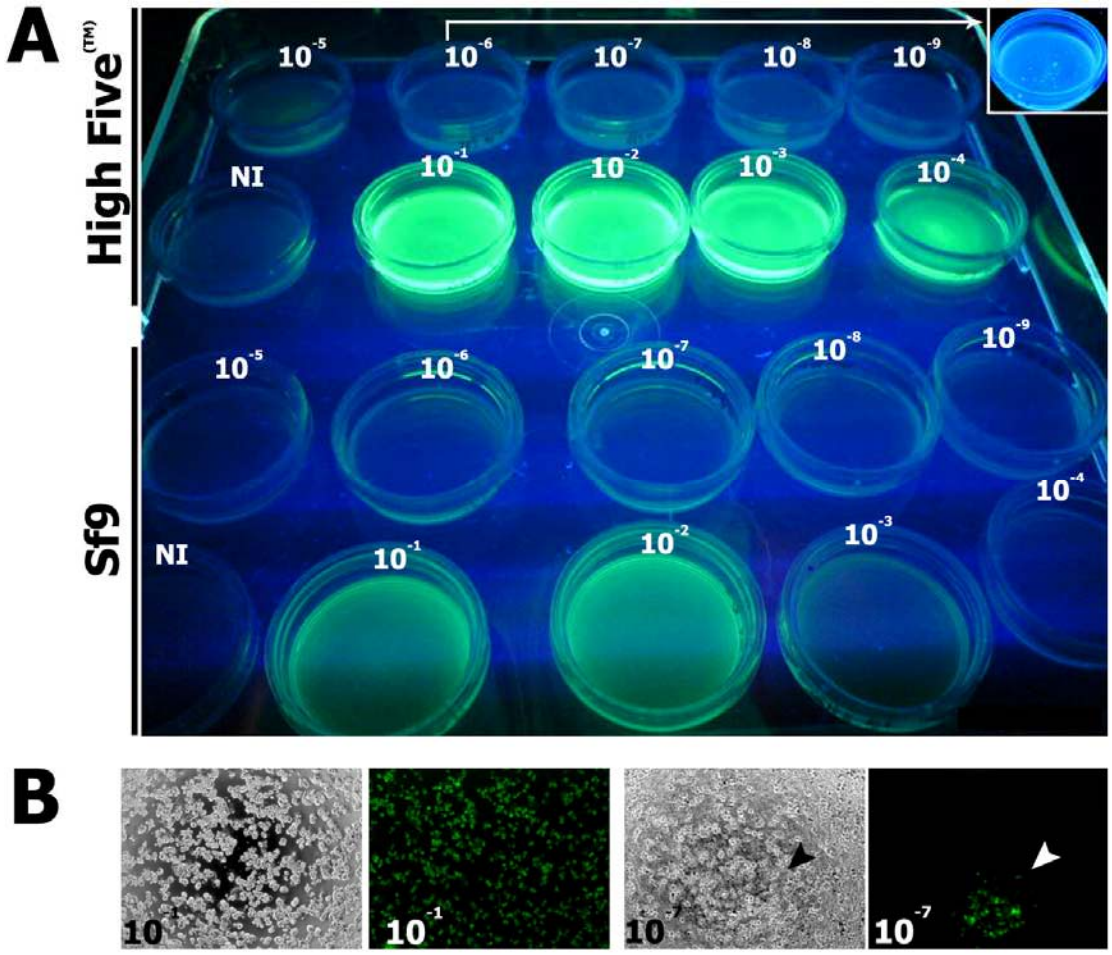



**Figura 3. Detecção de infecção por baculovirus através da expressão da EGFP. (A)** São mostradas placas do ensaio de placa para as linhagens Sf9 e High Five™ em diferentes concentrações de virus sobre um transiluminador UV. NI indica as placas controle não infectadas e os valores indicam o fator a diluição do estoque de vírus inicial. No Inset é possivel notar as várias colônias (*plaque forming unit* [pfu]). **(B)** Imagens das células das placas mostradas em A em microscópio invertido de fluorescência. A seta na diluição $10^{-7}$ indica uma 'plaque'.

Utilizando o pGreenBac a quantidade de vírus e de células infectadas puderam facilmente ser otimizadas e foi possível obter, secretados no meio de cultura, ~3 miligramas da proteína recombinante STC1 com tag de histidina C-terminal (STC1-HT) por litro de expressão. A purificação foi obtida por cromatografia líquida em três etapas a partir do meio de cultura como mostrado na Figura 4: primeiramente uma cromatografia de troca catiônica (SP Sepharose) seguida por uma cromatografia de afinidade a metal (Ni-NTA) e por último uma cromatografia de exclusão molecular (Superdex 200), sendo que a proteína foi então

mantida no tampão da cromatografia de exclusão molecular (60 mM MES, 600 mM NaCl pH 6,5) à 4°C.

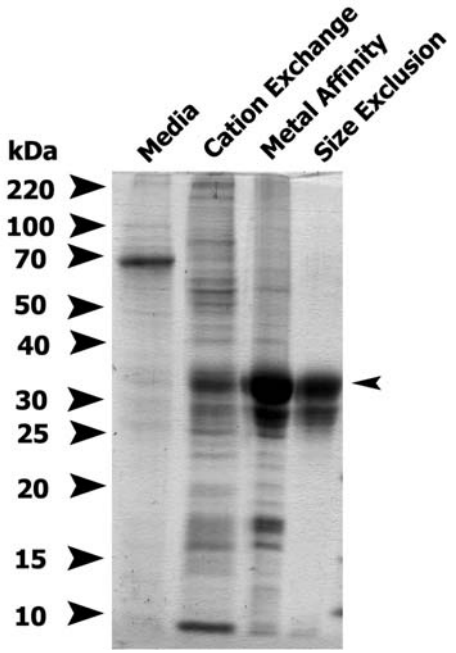



**Figura 4. Expressão e purificação da proteína recombinante STC1-HT utilizando o sistema de baculovírus.** SDS-PAGE de amostras do meio de cultura pós-infecção, e dos eluatos das cromatografias de troca iônica, de afinidade a metal e de exclusão molecular. Cabeça de seta à direita indica tamanho esperado da proteína recombinante expressa. Tamanhos indicados à esquerda são relativos ao marcador de proteína BENCHMark ladder

## 2. *Cristalização e análises inicias de difração de STC1*

De posse da proteína pura e em quantidades suficientes para estudos estruturais foi realizado um screening inicial de cristalização utilizando-se a proteína a 1 mg/mL em (10 mM MES, 100 mM NaCl pH 6,5). Foram testadas 544 condições pelo método de difusão de vapor em gota sentada. Foram obtidos cristais de proteína em forma de bastonete alongado (Figura 5) em três condições:

**3F10** [100mM Bis-tris propano pH 6,5; 20% PEG3350, 200mM $Na_2HPO_4/KH_2PO_4$];

**3F01** [100mM Bis-tris propano pH 6,5; 20% PEG3350, 200mM NaF]; e

**4C11** [100mM Bis-tris pH 5,5; 25% PEG3350; 200mM $MgCl_2$]).

Apesar dos cristais submetidos a difração de raios X serem pouco volumosos (não ideal para nossas condições de difração presentes no LNLS) e provenientes de screening inicial foi possível coletar dados de difração com resolução máxima de ~3,5 Å (Figura 6). Todavia, não foi possível a indexação dos dados devido irregularidade do padrão de difração e valores de $I/\sigma<I>$. pouco significativos.

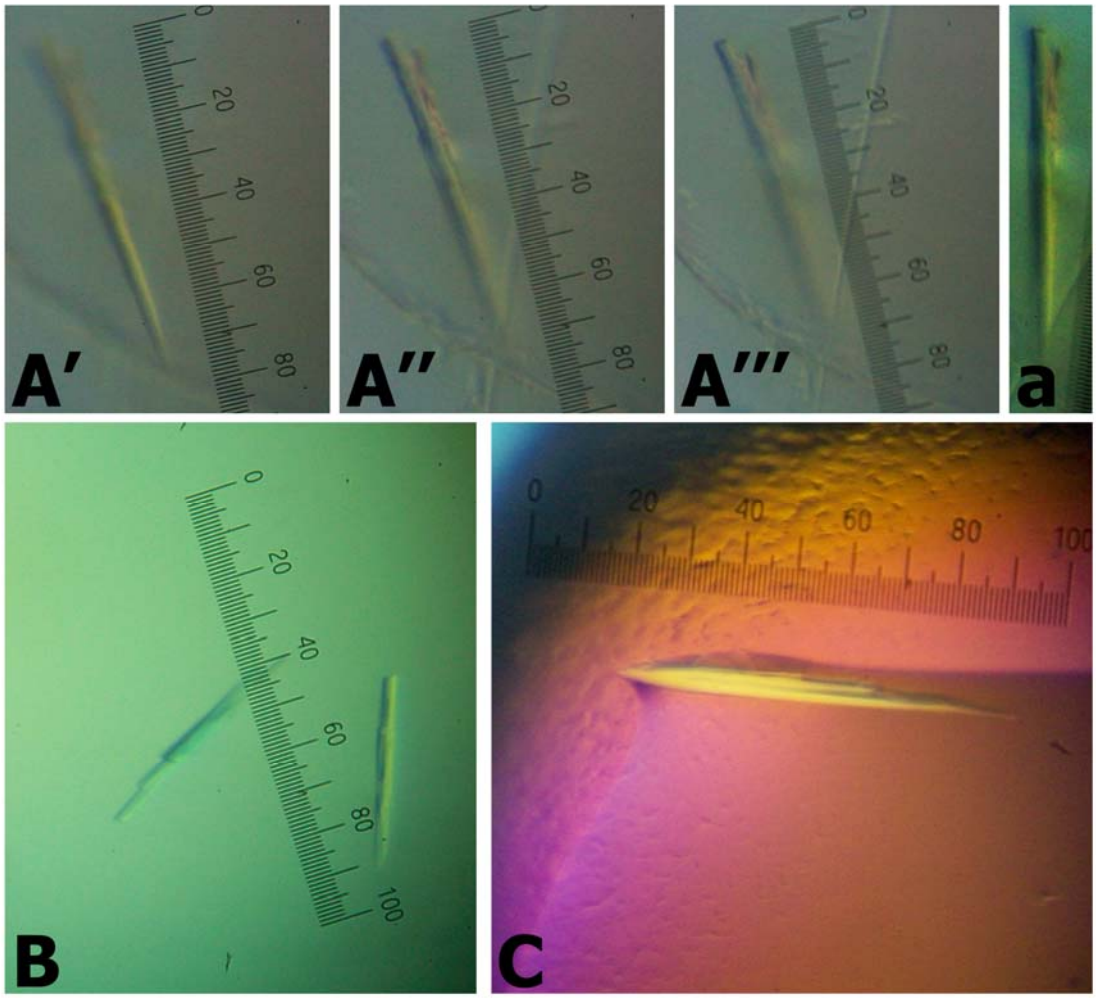



**Figura 5. Cristais da STC1-HT submetidos a difração de raios-X.** São apresentadas fotos de três condições diferentes de cristalização, os quais foram submetidos a difração de raios X. As imagens estão em ordem de melhor qualidade de difração para pior. (A', A" e A"') fotos do cristal obtido na condição de cristalização 3F10 [100mM Bis-tris propano pH 6,5; 20% PEG3350, 200mM $Na_2HPO_4/KH_2PO_4$] em diferentes planos de foco e em (a) Foi feita uma sobreposição das imagens A', A" e A"'. (B) foto dos cristais obtidos na condição de cristalização 4C11 [100mM Bis-tris pH 5,5; 25% PEG3350, 200mM $MgCl_2$]. (C) foto do cristal obtido na condição de cristalização 3F01 [100mM Bis-tris propano pH 6,5; 20% PEG3350, 200mM NaF]. As condições de cristalização estão descritas no texto. Escala 1 unidade = 5μm.

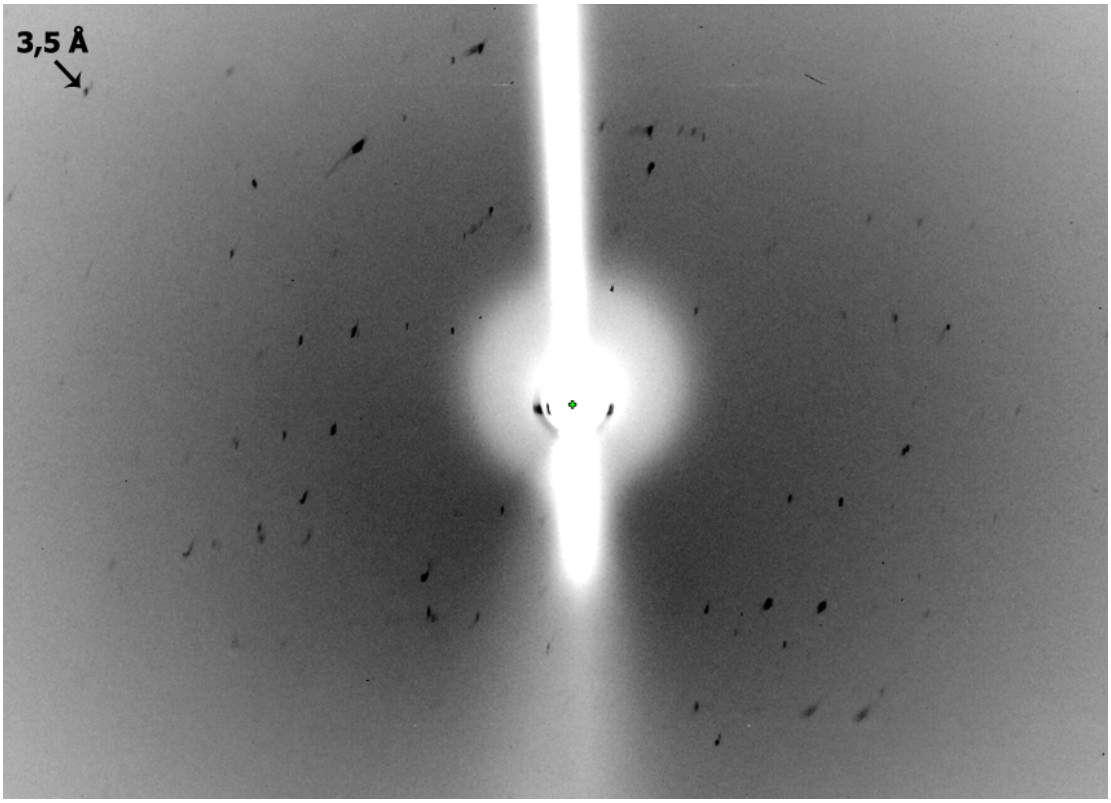



**Figura 6. Padrão de difração de raios X do cristal obtido na condição 3F10.** O cristal difratou a resolução de 3,5 Å utilizando um comprimento de 1,45 Å na linha de luz MX2 do Laboratório Nacional de Luz Síncrotron.

# VI. Conclusões e Perspectivas futuras

Ao final deste estudo conseguimos selecionar e validar STC1 como uma proteína marcadora de microambiente de leucemia infantil, e dar início a estudos funcionais e estruturais desta proteína. Apesar do pequeno tamanho amostral (n=3) dos nossos dados de validação por qPCR, recentemente STC1 foi encontrada em analises de secretoma em fibroblastos associados à melanma bem como em fibroblastos de mieloma múltiplo, contudo, não foi encontrada em fibroblastos sadios, leucócitos, células endoteliais e epteliais (Paulitschke *et al.*, 2009), reforçando assim nossos resultados. Além disso, Block e colaboradores (2009) também demonstraram que células multipotentes do estroma secretam STC1 com o objetivo de reduzir a apoptose em células lesionadas, assim como em células epiteliais de câncer de pulmão.

O processo de produção da proteína recombinante STC1-HT foi grandemente facilitado pelo uso do sistema por nós modificado de baculovírus, tendo-se em vista a, já previamente citada, dificuldade de titulação bem como de otimização da proteína nesse sistema. Ademais, a obtenção da proteína nesse sistema nos permitiu realizar análises espectroscópicas, bem como a caracterização estrutural em solução (SAXS) da proteína STC1-HT. Os dados obtidos nos estudos espectroscópicos estão em acordo com as análises *in silico* realizadas, bem como o padrão intercalado de pontes dissulfeto, que são típicos de proteínas secretadas, que por sua vez são compactas, como o envelope compacto e levemente alongado observado nas análises de SAXS. Todas as evidências estruturais aqui apresentadas apontam para informações sobre a composição da forma STC50 de STC1, e, embora estes não indiquem uma solução para a existência da forma bigSTC, deixam em aberto possibilidades para a resolução de tal problema, como por exemplo a predição de sítios de sumoilação na proteína STC1 o que resultaria no tamanho esperado do monômero da forma bigSTC.

Cabe salientar ainda que os *screenings* por duplo híbrido em levedura resultaram na recuperação de uma amostra significativa das proteínas que interagem com STC1 e que, interessantemente, localizam-se em locais onde STC1 já havia sido encontrada. Podemos citar, por exemplo, as proteínas mitocondriais que participam da fosforilação oxidativa, processo este que STC1 também foi implicada, bem como as proteínas nucleares que abrem um novo campo a ser explorado, uma vez que apesar de ter sido encontrada em núcleo de células alveolares da glândula mamária nenhuma função foi atribuída à STC1 neste compartimento. Além disso, o fato de encontrarmos uma das proteínas chave da resposta à proteínas desenoveladas, ERN1, dentre as interações vem reforçar o papel desta família de proteínas como sensores de stress podendo proteger bem como levar à morte celular. De um modo geral nossas análises mostram, como vem cada vez mais sendo mostrado na literatura, que STC1 é uma proteína multifuncional atuando em diversos processos celulares dentro do núcleo, mitocrôndria e extracelularmente,

bem como, mostra-se claramente envolvida em processos essências para o desenvolvimento e progressão da leucemia.

São necessários e pertinentes mais estudos aprofundados com o intuito de esclarecer as vias e o modo de ação da STC1 em todos estes processos como, por exemplo, o modo de internalização ou de re-direcionamento da proteína da via de secreção para dentro da mitocôndria e núcleo, bem como estudos mais aprofundados sobre a estrutura desta proteína que possam possibilitar o desenho de drogas contra esse marcador de microambiente de leucemia. Pretendemos agora então realizar estudos cristalográficos para STC1, além de estender os estudos ao outro membro da família das STCs, STC2, e buscar por mais evidências de quais modificações possam levar à formação da forma bigSTC; bem como direcionar esforços na caracterização de um potencial receptor, e quem sabe um mecanismo de internalização ou redirecionamento de STC1 para outras vias que não a de secreção.

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# VIII. ANEXO

**DECLARAÇÃO**

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada *"Estudos estruturais e funcionais da proteína stanniocalcina-1 humana, um novo marcador de microambiente de leucemia"*:

( ) não se enquadra no § 3° do Artigo 1° da Informação CCPG 01/08, referente a bioética e biossegurança.

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

( ) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n°_____);

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°_____);

( X ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar: Comissão Interna de Biossegurança da ABTLus – Associação Brasileira de Tecnologia de Luz Síncrotron (Processo n° JK07.01).

_____
Aluno: Daniel Maragno Trindade

_____
Orientador: Jörg Kobarg

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

_____
Nome:
Função:

Profa. Dra. HELENA COUTINHO F. DE OLIVEIRA
Presidente
Comissão Interna de Biossegurança
CIBio/IB - UNICAMP

Uso exclusivo da CIBio:

Número de projeto / processo: 7K 07.01

**Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança da ABTLuS – Associação Brasileira de Tecnologia de Luz Síncrotron**

Título do projeto: SELEÇÃO E VALIDAÇÃO DE PROTEÍNAS MARCADORAS DE LEUCEMIA INFANTIL PARA ESTUDOS ESTRUTURAIS E FUNCIONAIS

Pesquisador responsável: Jörg Kobarg

Experimentador: **Daniel Maranho Trindade**

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [ x ]-doutorado, [ ]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto:

Apesar dos avanços das últimas décadas, que têm possibilitado melhorias significativas no diagnóstico e tratamento das crianças com câncer, alguns tipos dessa doença ainda são refratários ao tratamento. Grande parte deste sucesso terapêutico foi derivado da implantação de protocolos investigacionais multicêntricos, que permitiram maior racionalização na definição das estratégias quimio, radioterápica e cirúrgica. A disponibilidade de novas ferramentas biológicas, químicas e físicas, como por exemplo o seqüenciamento do genoma humano, a geração de anticorpos monoclonais como ferramentas moleculares, os métodos de análise de proteínas e ácidos nucléicos, e a síntese de bibliotecas de drogas através da química combinatorial, abrem novas possibilidades ao melhor entendimento do câncer, ao diagnóstico mais preciso, à avaliação molecular da eficácia do tratamento, como também à obtenção de novas drogas ou imunotoxinas baseadas em anticorpos. No presente projeto propomos selecionar e analisar marcadores moleculares da leucemia infantil através de trabalho integrado, visando utilizar as capacidades técnico-científicas complementares do Laboratório Nacional de Luz Síncrotron e do Centro Infantil Boldrini. Os objetivos principais deste projeto de doutorado incluem: 1) a seleção e "validação" de marcadores moleculares candidatos da literatura e dos bancos de dados, 2) a caracterização funcional e estrutural de 1 marcador tumoral protéico "validado".

A CIBio analisou este projeto em reunião realizada no dia: 27.08.2007 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários abaixo:

_____

_____

Presidente de CIBio - ABTLuS
Prof. Dr. Jörg Kobarg

Membro da CIBio - ABTLuS
Prof. Dr. Celso Eduardo Benedetti

Membro da CIBio - ABTLuS
Prof. Dr. Nilson Ivo Tonin Zanchin