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CTNNB1 syndrome: a pilot study

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ABSTRACT

CTNNB1 syndrome is a novel syndrome that is caused by the complete or partial deletion of one allele of *CTNNB1*. Clinical features of the syndrome include developmental delay, intellectual disability (ID), speech impairment, autistic behaviors, microcephaly and reduced motor capabilities, as well as characteristic craniofacial features and ocular anomalies, for example, vitreoretinopathy and retinal detachment[1-11]. The overall aim of this pilot study is to explore novel potential treatments. In particular, we will select small molecules from a library that we designed to stabilize the interaction between β–catenin and N-cadherin, which we have previously shown to be decreased in the *Batface* (*Bfc*) mouse, a preclinical model of *CTNNB1* syndrome[3]. Moreover, we will use proteomic approaches, accompanied by the characterization of metabolites and lipids, which can generate insights and indications for drug discovery. We will also test a novel programmable epigenetic editing technology, designed in one of our labs, to modulate the expression of possible off-targets misregulated by the identified molecules.

The project includes three main activities: 1) Molecules in the library will be screened in neurons cultured *in vitro* to test their efficacy in correcting the structural and functional changes caused by impaired β-catenin interactions with N-cadherin. The effect of the administrated molecules will be tested, taking into account the crucial role of the signalling pathway in which the β-catenin is involved, and we will modulate any possible off-target with a target-specific epigenetic writer. 2) The molecules and the epigenetic writers that show modulatory effects in cultured *Bfc* neurons will be tested in neurons differentiated from iPSCs that are isolated from patients with *CTNNB1* syndrome. 3) Then, we will select the treatment that shows the highest efficacy and most minor toxicity from the *in vitro* screening, and we will perform molecular analysis of proteins, metabolites, and lipids combined with a thorough functional study that will help define potential drug targets as well as monitoring of its efficacy by features critically associated with disease and treatment.

LAY ABSTRACT (*IN ITALIAN*)

La sindrome CTNNB1 è una nuova sindrome causata dalla delezione completa o parziale di un allele del gene CTNNB1. Le caratteristiche cliniche della sindrome includono ritardo dello sviluppo, disabilità intellettiva (ID), compromissione del linguaggio, comportamenti autistici, microcefalia e capacità motorie ridotte, nonché caratteristiche craniofacciali e anomalie oculari, ad esempio vitreoretinopatia e distacco della retina[1-11]. Lo scopo generale di questo studio pilota è quello di identificare nuovi possibili trattamenti. In particolare, selezioneremo molecole da una libreria che abbiamo progettato per stabilizzare l'interazione tra β-catenina e N-caderina, poichè abbiamo precedentemente dimostrato essere ridotta nel topo Batface (Bfc), un modello preclinico della sindrome CTNNB1[3]. Inoltre, utilizzeremo approcci di proteomica, accompagnati dalla caratterizzazione di metaboliti e lipidi, che possono generare approfondimenti e indicazioni per la scoperta di nuovi farmaci. Utilizeremo anche una nuova tecnologia di editing epigenetico programmabile, progettata in uno dei nostri laboratori, per modulare l'espressione di possibili *off-target* malregolati dalle molecole identificate.

Il progetto comprende tre parti principali: 1) Le molecole saranno studiate in neuroni mantenuti *in vitro* per validare la loro efficacia nel correggere i cambiamenti strutturali e funzionali causati dalle interazioni alterate della β-catenina con la N-caderina. Verrà studiato l'effetto delle molecole somministrate tenendo conto del ruolo cruciale della via di segnalazione in cui è coinvolta la β-catenina, e moduleremo ogni possibile *off-target* con un *writer* epigenetico diretto sul target genetico specifico. 2) Le molecole e i *writer* epigenetici che mostrano effetti modulatori nei neuroni Bfc in coltura saranno studiati ulteriormente in neuroni differenziati da iPSC isolati da pazienti con sindrome CTNNB1. 3) Ins eguito selezioneremo il trattamento che mostra la massima efficacia e la minor tossicità dallo screening *in vitro*, ed eseguiremo alcune analisi specifiche delle proteine, dei metaboliti e dei lipidi combinate con uno studio funzionale approfondito che ci permetterà di definire potenziali bersagli farmacologici così come il monitoraggio della sua efficacia da parte di caratteristiche criticamente associate alla malattia e al trattamento.

Risultati attesi: attraverso lo studio di una selezione di target molecolari, dal seguente progetto ci aspettiamo di identificare nuovi potenziali target per lo sviluppo di farmaci capaci di agire su processi cellulari anomali nella sindrome CTNNB1. Inoltre, lo studio sul modello animale ci permetterà di testare questi effetti in vivo e di valutare gli effetti comportamentali del target più promettente.

RELEVANT SCIENTIFIC BACKGROUND

CTNNB1 syndrome and preclinical advances. Loss-of-function mutations in *CTNNB1* that lead to ID were first reported in 2012[1]. Then, in 2014, our group identified *de novo* mutations within *CTNNB1* in patients with a recognizable syndrome with traits of autistic spectrum disorder[3]. In parallel, we developed a novel mouse line, the *Bfc*, that displays similar features to human patients[3]. In recent years, a few hundred patients have been identified worldwide, with an increasing number of cases reported [1-11]. *CTNNB1* is one of the 153 genes on the SPARK Gene List and is classified under Category 1 of ASD-risk genes in the SFARI Gene Database. Ctnnb1Bfc*/Ctnnb1*$^{+}$ (*Bfc*) mice, which carry a missense mutation in the *Ctnnb1* gene, show analogous phenotypes to the human syndrome, such as craniofacial features, motor deficits, neurodevelopmental abnormalities, cognitive deficits and neurological traits[3, 4]. Among the numerous phenotypes, *Bfc* adult mice also show reduced precision in cognitive tasks, such as interval timing, and pups present with reduced ultrasonic vocalization upon maternal separation[3, 12, 13]. In addition, a decreased clustering of synaptic vesicles in the hippocampal neurons was observed. Furthermore, *Bfc* hippocampal neurons show increased neurite outgrowth in early days *in vitro* (DIV), but lower dendritic branching at later DIV. Moreover, the mature network of hippocampal neurons *in vitro* shows a reduced long-term potentiation[3]. The mutation in *Bfc* is a missense mutation of C>A, which results in the substitution of Threonine 653 to Lysine. Mutation in *Bfc* occurs at a residue adjacent to Tyrosine 654 (Y654), which was shown to regulate the interaction with E-cadherin. Indeed, we demonstrated that the interaction between N-cadherin and β–catenin is reduced *in vitro* cultured neurons

isolated from *Bfc*[3]. N-cadherin is a synaptic adhesion protein that regulates both exo- and endo-cytosis of vesicles[14]. The cadherin–catenin junctions are highly dynamic molecular complexes. Loss of cadherin-mediated cell adhesion can promote β–catenin release and thus its signalling activity. On the other hand, cadherin can act as a membrane trap for a fraction of free β -catenin. Therefore, mutations laying in the e-cadherin binding portion of the β–catenin may determine the abnormal activation of signalling pathways rather than the physiological adhesion processes. Thus, in neurons, the Y654 residue of β–catenin was reported to be crucial in the relocalization of the protein to the synapses upon depolarization, where its interaction with N-cadherin modulates synaptic strength[15]. In addition, this process is independent of new protein synthesis but rather regulated by the phosphorylation status of Y654[15]. Furthermore, the interaction with N-cadherin is critical in regulating the release probability of synaptic vesicles in a trans-synaptic manner[14, 16]. Therefore, activity-induced relocalization of β–catenin and the subsequent interaction with N-cadherin is important in regulating synaptic strength, which is compromised in ASD[17]. However, the dissociation of the N-cadherin/β–catenin complex is necessary to increase synaptic vesicle mobility upon activating TrkB receptors by BDNF. Therefore, our screen will focus on quantifying calcium-dependent dendritic arborization after stimulation with KCl as a read-out of the efficacy, enabling us to focus on the molecules that can increase the formation of N-cadherin/β–catenin complex in re-stabilized synapses after the depolarization.

Programmable epigenetic writers. In recent years we witnessed a fast revolution in gene-editing technologies, such as clustered regularly interspaced palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins. CRISPR technology is based on DNA breaks and homology-directed repair mechanisms, which can be programmed by noncoding single guide RNAs (sgRNAs) to guide specific nuclease (e.g., Cas9 and all successively modified proteins) to induce site-specific DNA cleavage in effectively any cellular system and organism. Despite the potential of DNA editing in gene therapy, single-strand nicks bring significant cellular toxicity, currently preventing the large use of CRISPR-based applications in gene therapy. The relatively low efficiency of precise homology-directed repairing, the presence of off-target mutations, and the difficulties of introducing CRISPR nucleases and targeting templates into stem cells are common issues in exploiting CRISPR gene-editing to treat human diseases. To overcome these issues, it has been proposed to adapt CRISPR technology to control gene transcription epigenetic regulatory mechanisms. Among them, DNA methylation on CpG dinucleotides regulates chromatin's opening or closure, binding specific transcription factors to the gene promoter and assembling the transcription machinery. Aberrant methylation regulation at gene promoters causes many diseases, such as tumours and behavioural and cognitive disorders. Thus, sequence-specific methylation and demethylation editing would have significant clinical benefits. To date, only a few epigenetic drugs have been approved for human treatment in neurological diseases and cancers. However, current methods of manipulating DNA methylation are primarily based on global inhibition of DNA methyltransferases via small molecule compounds such as hypomethylating agents, including Azacitidine or Decitabine. These molecules behave as a false substrate that replaces the DNA cytosine with a similar nucleoside (e.g., Cytidine) that cannot be methylated. During DNA replication, the progressive substitution of cytosines leads to broad epigenetic changes and activation of endogenous retroviruses. As a consequence of this global constitutive methylation, side effects related to off-target organs or cell types throughout the whole organism are recurrent. In the alternative, it has been shown that by combining catalytically inactive Cas protein with parts of human DNMT[24] and bacterial methyltransferases[25] or human TET1 catalytic domains, the system can be used to drive the epigenetic writers on a specific sequence of the DNA.

Molecular analyses of proteins. It is our aim to discover and validate the effects of novel drug treatments on proteins. This approach will be pivotal to assess physiological functional recovery or correction in relation to the disease. We propose to use a platform of advanced technologies, second-level investigations, endowed with greater specificity related to the drug's mechanism of action. MS-based proteomics is increasingly a powerful and versatile tool for elucidating dysregulated mechanisms in disease and for monitoring drug action. The instrumentations available in the Core Facilities - Clinical Proteomics and Metabolomics laboratory of the G.

Gaslini Institute, together with available software and workflows are substantially mature and robust to achieve an applicative quantum leap in drug discovery. Although proteomics does not yet have sensitivity and coverage as RNA sequencing, it does provide a unique functional dimension directly relevant to the mechanistic information of disease and drug action. The further developments planned are oriented towards further increasing sensitivity as well as throughput, so as to allow the study of only a few or single cells, cellular heterogeneity, and subcellular and spatial mechanisms of drug and of disease molecular phenotypes. These developments are supported by computational concepts and new emerging software. Systematic embedding of such approaches into drug discovery projects will tremendously benefit hit-to-lead-to-candidate cycle times and may reduce late-stage attrition.

PRELIMINARY RESULTS

Prior to designing the library of interaction stabilizers, the three-dimensional structure of the complex between the wild-type β–catenin and N-cadherin human proteins (hereafter, wtCat/Cad) was modelled using the coordinates of the three-dimensional X-ray crystal structures that are deposited in the Protein Data Bank (PDB). In addition, the complex between the T653K β–catenin mutant in interaction with the E-cadherin (hereafter, T653KCat/Cad) has been created by replacement of the 653 Threonine residue with a lysine in the β–catenin sequence. WtCat/Cad and T653KCat/Cad complexes were then subjected to 300ns of molecular dynamics simulation in explicit water to study the dynamic and stability of the two models. Our trajectories analyzed the effect of the T653K mutation on the protein interactions. From each MD trajectory, representative complex states are extracted using a clustering approach and then subjected to alanine scanning analysis to identify the key residues responsible for stabilizing the interface. The results confirmed the residues reported in the literature and identified new ones. In addition, the comparative analysis identified the interface regions most affected by the T653K mutation and those having a suitable *druggability* profile.
Thus, a structured base approach on representative structures extracted from our trajectories was applied, and a virtual screening campaign of commercial databases was performed to find small molecules able to stabilize the complex, especially in the presence of the CatCad mutation. Of note, the structural modelling of β–catenin/N-cadherin complex has enabled an alanine scanning analysis *in silico*, which was focused on the C-terminal region (300-781 aa) of β–catenin, based on the abovementioned structures of wtCat/Cad. This analysis identified several residues that match or are adjacent to the residues mutated in clinical cases. This suggests the relevance of our approach also for other clinical cases that do not involve mutations in the proximity of Y654.

PROJECT DESCRIPTION

Molecules screening: We will test a library of small molecules to identify those that will stabilize the interaction between β–catenin and N-cadherin, an interaction decreased in our mouse model of CTNNB1 syndrome, Bfc. Therefore, here we will focus on developing our therapeutic approach to stabilizing the N-cadherin/β–catenin complex, which would enrich the pool of CTNNB1 locally at the synapses, rather than augmenting the total level of β–catenin, which poses a high risk for the oncogenic signalling that is mediated by β–catenin when it translocated in the nucleus. We have modelled three-dimensional structures of the human N-cadherin/β–catenin complex based on E-cadherin and β–catenin mouse protein structures available on Protein Data Bank (PDB). These models were analyzed by molecular dynamics to identify the targets for designing the small molecules for stabilizing the complex.
Screening of the library of molecules will be carried out by imaging analysis of *in vitro* primary cultured neurons from Bfc mouse embryos by testing those that can restore the deficiency in calcium-dependent dendritic arborization in Bfc. The molecules pre-selected from the *in vitro* screening will be subsequently confirmed by further biochemical analysis and electrophysiological analysis of Bfc cultured neurons. We will test the ability of the designed molecules to increase the N-cadherin/β–catenin coupling with a Bimolecular Fluorescence Complementation (BiFC)[23] assay for direct visualization of protein-protein interaction *in vitro*. Fusing the C-

terminal fragment of a green fluorescent protein with the N-cadherin and the N-terminal with the β–catenin, the fluorescence will be produced when the N-cadherin/β–catenin complex is formed. This represents a fast screening method to test the efficacy of the designed molecules to increase the binding of the proteins.
In addition, the analysis will be repeated in iPSCs isolated from the patients of CTNNB1 syndrome currently under development by Simons Searchlight (NY, USA) and by Children's Medical Research Institute (Sydney, Australia) to confirm the translational probability of this treatment. In the final stage of this project, a compound, which has shown the highest efficacy *in vitro*, will be used for cognitive and behavioural analysis in a mouse model of CTNNB1 syndrome *in vivo*.

Off-targets analysis. β–catenin is an integral structural component of cadherin-based adherens junctions and the key nuclear effector of canonical Wnt signalling in the nucleus. Imbalance in the structural and signalling properties of β–catenin often results in disease and deregulated growth connected to cancer and metastasis.
Therefore, the increase in N-cadherin/β–catenin complex formation rate may lead to the imbalance of nuclear signalling and consequent activation of pathophysiological pathways. In parallel to the molecule screening, we will test the activation of downstream β–catenin signalling pathways, such as the TGF-dependent (AXIN2, LEF1, SP5, DKK1) or TGF-independent (VGF, MMP13, ADAM family or GADD45) gene expression.
We will test the efficacy of a new target-specific epigenetic writer designed in our lab to modulate the possible misregulation of the β–catenin downstream pathways. Essentially, epigenetic modifications do not alter DNA sequences, and they bring with them the potential to bypass part of the negative effects associated with gene-editing. We developed a novel system in which we fused a modified catalytically inactive Cas (i.e., the small dCas12f), which is engineered as programmable and tissue-specific epigenome editing technology. We named this system Targeting Units for Specific Epigenetic Remodeling or TUSER. The scope of our invention (Priority patent application IT-102022000006287 and IT 102022000006296) was to provide a control system that can precisely and timely regulate the expression of target mammalian genes by rewriting their epigenetic landscape without introducing changes to the underlying DNA sequences.

Our overall goal is to use these two strategies to compensate for deficits in synaptic function of endogenous β–catenin in CTNNB1 syndrome by enriching the pool of β–catenin that interacts with N-cadherin, a neural adhesion protein at the synapses. Selection of molecules and/or programable epigenetic writers that enhance the formation of N-cadherin/ β–catenin complex at synapses upon depolarization, but those that still enable the endogenous processes of phosphorylation and dephosphorylation on β–catenin, should improve the impaired synaptic transmission by increasing the readily releasable pool and by maintaining β–catenin at the synapses for activity-dependent regulation of dendritic spines.

Proteomics-based screening.
Protein-protein interactions (PPI). Here the goal is to discover protein interactors by affinity mechanisms. The classical approach for PPI identification captures direct and indirect protein interactants through immunoprecipitation or affinity purification of single endogenous or epitope-labeled proteins. The members of protein complexes can be profiled in the absence or presence of a drug, which allows for the identification of compound-sensitive PPIs and the exploration of mechanisms by which a compound modulates the activity of the target. This is relevant for molecules designed to inhibit specific PPIs and also provides information on the mechanisms and selectivity of the complex for small molecules targeting the catalytic subunits of multiprotein complexes. The distinction between specific and non-specific PPIs is fundamental for the interpretation of interaction proteomics experiments. Analytical strategies for this purpose include enrichments with more than one antibody or protein bait against a target, negative controls with isotype match, as well as experimental and in silico delineation of co-enriched contaminants.
LIP–MS - to identify altered protein folding by compound binding by altered protease susceptibility. Small molecule-protein interactions control many cellular processes and "limited proteolysis mass spectrometry" (LiP-MS) can be used to study them even in a complex sample. In this approach, a small molecule, such as a drug, is

added to a cell lysate (in our case, the same cells and drugs as the drug screening experiment could be used), to be subsequently incubated with a non-specific protease such as proteinase K. Since the binding of a small molecule can locally alter the accessibility of the protease to its protein target, peptide fragments specific to each condition are produced. The fragment size is then reduced by second trypsin digestion to be then measured using a traditional bottom-up proteomics approach. The abundance of the different peptides produces structural imprints for all detected proteins. This information allows us to identify with resolution at the peptide level the site of drug-protein interaction as well as highlight any off-target. This type of analysis is also synergistic and easily integrated with more traditional approaches such as differential analysis, performed on a part of the same cell lysate but not treated with proteinase K, in order to identify dysregulations of the cellular pathways, or protein affinity analysis for to study protein-protein interaction networks and how these can change following drug treatments. Finally, the combination of these three protein characterizations, structure, dysregulation and interaction, is a valid aid in the mechanistic characterization of drug treatment in the cell model.

Global phosphoproteomics - to map kinase and substrate modifications in terms of activity and stability. Most intracellular signaling pathways are regulated by the dynamic modification of proteins through PTMs. Proteomics approaches for the complete detection of stable PTM-modified peptides are well-established and can be used to monitor drug-induced signaling changes. Since most dynamic PTMs are substoichiometric (they only occur on a subgroup of proteins), these workflows usually include enrichment stages using matrices with appropriate physical properties (such as positively charged state or hydrophobicity due to the enrichment of phosphopeptides) or antibodies. Therefore, they may require a greater amount of biological starting material than expression proteomics experiments. Specifically, in our proposed technology platform, global phosphoproteomics can be used for to systematically detect the activation or inhibition of signaling pathways downstream of drug treatment. As such, the approach may lead to the identification of drug-activated or inhibited kinase substrates. As an example, we highlight the frequency with which drug resistance in cancer is often associated with compensatory rewiring of signaling. In this context, phosphoproteomics can define adaptivekinase responses to single-drug therapies or evaluate combinations of drug treatments.

Regulome - Highly Parallel Quantification and Compartment Localization of Transcription Factors and Nuclear Proteins. The Regulome is the machinery that interprets our DNA — ensuring the right genes are turned on at the right time, in the right cells. Regulome dysfunction is a primary cause of cancer as well as many other diseases. Transcription factors and other chromatin-associated proteins are difficult to quantify comprehensively. Here, we can combine facile nuclear sub-fractionation with data-independent acquisition mass spectrometry to achieve rapid, sensitive, and highly parallel quantification of the nuclear proteome in human cells. The method is simple and enables system-level study of previously inaccessible chromatin and genome regulators.

EXPERIMENTAL METHODS

We will use quantitative imaging analysis to screen up to 30 compounds in a co-culture of wt and +/*Bfc* neurons *in vitro*. We will apply the BiFC assay to asses the T653KCat/Cad interaction in response to the treatments. Moreover, we had previously shown a dramatic reduction in the number of neurites in depolarized *Bfc* neurons compared to that in depolarized wt neurons[3]. Briefly, primary neurons isolated from +/*Bfc* mice will be transfected with pCAG-GFP, whereas the neurons from wt will be electroporated with pCAG-mCherry. Both types of neurons are pooled and plated onto coverslips together. The response in dendritic arborization upon depolarization with KCl stimulation will be used as a read-out for the efficacy of the compounds in increasing the N-cadherin and β–catenin [18, 19].

The molecules found to be effective in increasing *Bfc* neuron dendritic arborization will subsequently be examined further to confirm their action on the interaction between N-cadherin and β–catenin by co-immunoprecipitation. The efficacy of the pharmacological treatment on the docked synaptic vesicles will be visualized by electron microscopy conducted by our IIT-EM facility. In addition, electrophysiological measurements of neuronal activity by microelectrode array (MEA) will assess their efficacy for increasing synaptic transmission. Furthermore, the most effective compounds will be tested in induced pluripotent stem cells (iPSCs) isolated from *CTNNB1* patients compared to an iPSC line from healthy control subjects. The iPSCs will be differentiated into neurons and

subjected to MEA electrophysiological studies and co-immunoprecipitation of N-cadherin and β-catenin. There are several options for patient-derived iPSCs for our proposed project. One that was recently published[20], and four lines of iPSCs currently under development, three by Simons Foundation. In addition, another line by Children's Medical Research Institute (Sydney, Australia) is currently under preparation.

The most promising compounds will be selected from the screening *in vitro* to be tested for their effect on improving the cognitive and behavioral phenotypes of *Bfc* mice. A selection of primary and secondary behavioral screens will be applied.

In detail:

(i) Screening of the library in *Bfc* neurons cultured *in vitro* and the statistical analysis.

This part includes: a high-throughput imaging analysis of primary neurons cultured from *Bfc* and *wt* embryos treated with the novel/repurposed compounds, the off-targets analysis and the possible application of TUSER technology to modulate the altered gene expression

Expected outcome: refinement of the library compounds and refinement of the terapeutical strategy.

(*ii-a*) – MOUSE: tests of the selected molecules in *Bfc* neurons cultured *in vitro*:

1) interaction between N-cadherin and β–catenin by co-immunoprecipitation.
2) quantification of docked synaptic vesicles in the treated *Bfc* neurons by electron microscopy
3) network activity of the treated *Bfc* neurons *in vitro* by MEA.

Expected outcome: assessment of the efficacy of the library selection in cultured mouse neurons.

(*ii-b*) – HUMAN - Molecular tests in iPSCs from CTNNB1 syndrome patients:

1) interaction between N-cadherin and β–catenin in iPSCs by co-immunoprecipitation.
2) network activity of the treated "neurons" (differentiated from iPSCs).

Expected outcome: assessment of the efficacy of the library selection in patient-derived cells.

(iii) Behavioral tests in two preclinical mouse models:

1) *Bfc* and control mice will be treated with the selected compound and tested in primary and advanced cognitive automated protocols developed in our lab[21].

Expected outcome: *in vivo* assessment of the selected molecule in the two mouse models of *CTNNB1* syndrome.

(iv) small molecule-protein interaction studies:

1) First(Omics, Glogal Phosphoproteomics)- and second-level investigations(PPI, LiPS, and Regulome) on treated biological models.

Expected outcome: Molecular correlation of drug effects on the cellular models. Identification of signal transduction mechanisms as well as any pharmacological off-target.

Pitfalls and alternative strategies

This project aims to identify a compound that can compensate for the deficiency in N-cadherin/β–catenin interaction. However, we are aware that the strengthening of this interaction brings with it a risk of worsening synaptic plasticity. To address this issue, we are designing molecules that will still enable the phosphorylation of endogenous β–catenin. Another possible pitfall is if the differences between treatment and control are not distinct sufficiently to a level that can be easily quantified. Should we encounter such technical difficulty, the quantity of arborization can be improved by comparing the phenotypes of three conditions that can be used as a scale of effects. The first on the positive scale is the neurons that overexpress unphosphorylated (Y654F) β–catenin[15], followed by the neurons treated with ACY-1215 (aka Ricolinostat) to increase β–catenin membrane localization [22] as the second positive control, whereas neurons that overexpress a phosphomimick (Y654E) β–catenin, and untreated +/*Bfc* neurons are the negative controls on the scale.

Haploinsufficiency of β–catenin has been reported in lymphocytes derived from patients. It will be critical to test if the stabilization of the N-cadherin/β–catenin complex in patient-derived cells can lead successfully to an increased synaptic response when the total pool of intracellular β–catenin is reduced. As an alternative strategy in the patient-derived cells, we could virally express the Y653F mutant of β–catenin, which remains in an unphosphorylated state that favours its interaction with N-cadherin, under a neuronal-specific promoter, and combine this expression of β–catenin with a treatment of our selected compounds to stabilize the interaction with N-cadherin. Another possible pitfall to consider during our proposed project is the possible toxicity of the small molecules to be tested. The toxic effect of the treatment on the cultured neurons will be assessed by quantifying the integrity of the neuronal network, by canonical cell viability assay, and evaluating the mitochondrial depolarization as an early sign of cytotoxic damage by novel immunofluorescence techniques (e.g., JC-10).

Project significance and relevance to CTNNB1 syndrome

The uniqueness of this project is that the molecular alterations in mice models of *CTNNB1* syndrome have not yet been studied for therapeutic purposes. As described above, the mouse models demonstrate numerous phenotypes that remarkably resemble the clinical descriptions of patients with CTNNB1 syndrome.

Furthermore, the eventual therapeutic approach to be developed from our proposed project would potentially improve the cognition of the current patients, as the treatment can be applied at any point in their lifetime. Pharmacological intervention or viral-based epigenetic drugs implies an easier and safer application in comparison to a more complex treatment such as gene replacement or viral delivery of β–catenin. Of note, AAV-delivery of β–catenin would require close monitoring to ensure that the overexpressed level of β–catenin does not lead to oncogenic signalling.

Specific behavioral analysis of mouse mutants from our study will provide an insight into the putative therapeutic use of the compounds identified in our screening in treatment for behaviors that are characteristic not only in CTNNB1 syndrome but also for overlapping behaviors in ASD.

Provisions concerning data protection

The Fondazione Istituto Italiano di Tecnologia and the IRCCS Istituto Giannina Gaslini expressly declare to be informed and to agree that personal data provided during the execution of this project will be processed solely for the purposes of the Project and, in any case, in compliance with all the rules of the Regulation (EU) 2016/679 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data (hereinafter GDPR).

In particular, the Parties acknowledge that, according to the principles enunciated in Article 5 of GDPR, personal data shall be:

a. processed lawfully, fairly and in a transparent manner in relation to the data subject;

b. collected for specified, explicit and legitimate purposes and further processed in a manner that is compatible with those purposes;

c. adequate, relevant and limited to what is necessary in relation to the purposes for which they are processed;

d. accurate and, where necessary, kept up to date through the adoption of every reasonable step to erase or to rectify without delay the personal data that are inaccurate;

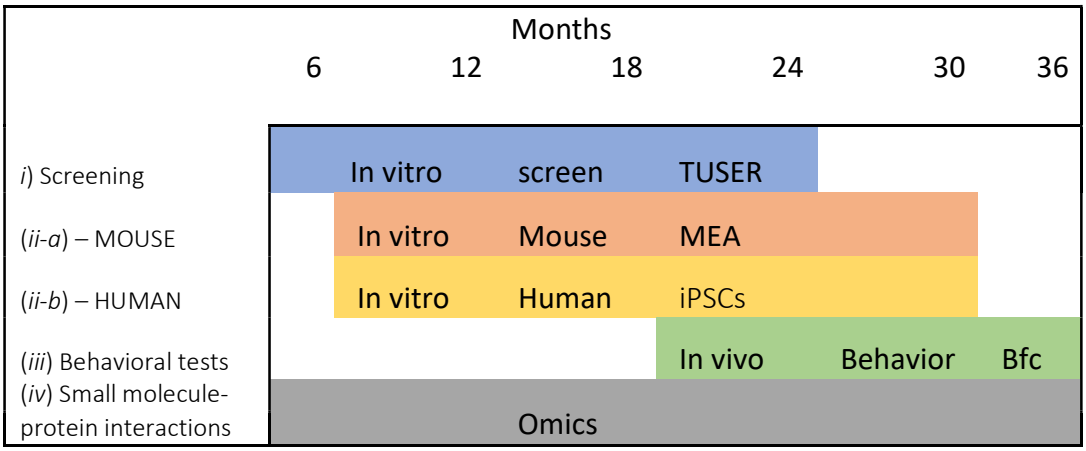
e. kept in a form which permits identification of data subjects for no longer than is necessary for the purposes for which the personal data are processed;

f. processed in a manner that ensures appropriate security of the personal data, including protection against unauthorized or unlawful processing and against accidental loss, destruction or damage, using appropriate technical or organizational measures.

PROJECT IN SHORT AND BUDGET INFORMATION

PROJECT DURATION: 3 years

TIMING – GANNT CHART:



BUDGET (in euros)

Gaslini		year 1	year 2	year 3	TOT.	
	Post-doc	40000	40000	40000		*120000*
	Consumables	10000	10000	10000		*30000*
	Service	10000	10000	10000		*30000*
IIT						
	Post-doc	40000	40000	40000		*120000*
	Consumables	10000	10000	10000		*30000*
	iPS study	8000	8000	8000		*24000*
	Mouse costs	8000	8000	8000		*24000*
		126000	*126000*	*126000*	*Tot. (euros)*	***378000***

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