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A cytoplasmic role of Wnt- β -catenin transcriptional cofactors in tooth enamel formation

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Abstract: Wnt- β -catenin signaling is necessary for the development of virtually all organs, including teeth. Bcl9 and Bcl9l are important β -catenin transcriptional co-factors. In the nucleus, they simultaneously bind β -catenin and the transcriptional activator Pygo2 in order to activate Wnt-target gene transcription. Here we report an entirely new function of Bcl9, Bcl9l and Pygo2. We show that, in tooth development, they are not required for Wnt- β -catenin-dependent transcription. However, Bcl9, Bcl9l and Pygo2 are localized mainly in the cytoplasm of the epithelial-derived ameloblasts, the cells responsible for enamel production. In ameloblasts, Bcl9 interacts with proteins involved in enamel formation, and its conditional deletion leads to teeth with defective enamel, a condition strikingly reminiscent of human enamel pathologies. Overall, our data reveal that these β -catenin transcriptional co-factors fine-tune late differentiation events during odontogenesis through a previously unknown cytoplasmic function, revealing that their roles go beyond the paradigmatic view previously accepted.

One-sentence summary: An unexpected non-transcriptional, cytoplasmic activity of Bcl9/9l is required for tooth enamel fine-tuning.

Introduction

Wnt signaling plays fundamental roles in virtually all aspects of embryonic development and organogenesis (1). Wnt signaling-dependent transcription is mediated by nuclear β -catenin, which binds to the TCF transcription factors and serves as a scaffold for an ensemble of cofactors, such as Lgs and Pygopus, important for transcriptional activation (2). Lgs and Pygopus were first identified in *Drosophila melanogaster* as dedicated Arm (the fly β -catenin) cofactors. Their mutation induced a phenotype recapitulating a complete loss of Wnt signaling (3, 4). A “chain of adaptors” model was proposed, in which the four proteins, TCF, β -catenin, Lgs, and Pygopus are successively recruited to the DNA in order to activate Wnt-target gene expression (5–7). In this model, the only function of Lgs is to recruit Pygopus to β -catenin. However, mutations of the Pygopus homologs (*Pygo1* and *Pygo2*, hereafter referred to as *Pygo1/2*) and of Lgs homologs (*Bcl9* and *Bcl9l*, referred to as *Bcl9/9l*) in the mouse revealed that their phenotype does not always recapitulate the effects of Wnt- β -catenin loss (8–10). These proteins are therefore considered as tissue-specific β -catenin transcriptional effectors (8, 10).

One organ whose development is critically dependent on the Wnt- β -catenin pathway is the tooth (11–13). Wnt ligands are expressed in developing teeth, and loss of β -catenin leads to the arrest of odontogenesis at early stages (11). Teeth develop as a result of sequential and reciprocal interactions between cells of the oral epithelium and cranial neural crest-derived mesenchymal cells (14). Odontogenesis proceeds through a series of well-defined morphological stages, namely bud, cap, bell, and differentiation/mineralization, both in rodent and humans (14). Differentiation of mesenchymal cells gives rise to the dentin-producing odontoblasts, while the enamel-forming ameloblasts originate from epithelial cells (14). In contrast to molars, rodent incisors are continuously growing organs with anatomically distinct and molecularly defined territories where cell proliferation, differentiation, and maturation events can be easily analyzed. A pool of dental epithelial stem cells located in the proximal part of the incisor (labial cervical loop) ensures the constant generation of ameloblasts. These cells produce new enamel, compensating hard tissue loss due to mastication. The Wnt- β -catenin signaling has been shown to be a key regulator of this process (13).

Here we investigate the role of *Bcl9/9l* and *Pygo1/2* during tooth formation and homeostasis using the mouse incisor as model system. We find that *Bcl9/9l* and *Pygo1/2* are strictly required for amelogenesis (the formation of the enamel) and that their role is not mediated by the Wnt- β -catenin pathway. Interestingly *Bcl9/9l* and *Pygo1/2* are acting in the cytoplasm along the secretory pathway, where they interact with the enamel-specific protein amelogenin, a crucial actor in amelogenesis. We speculate that *Bcl9/9l* and *Pygo2* might also be key factors important for the secretion of specific proteins in other tissues.

RESULTS

***Bcl9/9l* and *Pygo2* are expressed in the secretory, differentiated ameloblasts**

We first analyzed the expression pattern of β -catenin, *Bcl9*, *Bcl9l* and *Pygo2* in the developing mouse incisor (Fig. 1A–C) by immunohistochemistry. β -catenin expression is detected only in the epithelial component of the incisor, from the cervical loop area to the most differentiated anterior part (Fig. 1D–F, S1). Similarly to β -catenin, *Bcl9* is expressed in dental epithelial cells at embryonic day 15.5 (E15.5, Fig. 1G) and postnatally at day 0 (P0, Fig. 1H).

Interestingly however, at P0, Bcl9 is restricted to differentiated ameloblasts, where it displays an asymmetric cytoplasmic distribution towards their apical secretory part (Fig. 1H, J, S1). Bcl9l is expressed at E15.5 both in the epithelial as well as in the mesenchymal cells (Fig. 1J), but, at P0, its expression appears limited to the dental epithelium – also Bcl9l is predominantly cytoplasmic (Fig. 1K, L). Similarly, Pygo2 is present at E15.5 in both epithelial and mesenchymal cells (Fig. 1M), but at P0 its expression is observed only in the cytoplasm of epithelial cells, most notably ameloblasts and the underlying stratum intermedium (Fig. 1N, O, S1).

Conditional deletion of β -catenin, but not of Bcl9/9l, leads to arrested tooth development

The K14-Cre-driven epithelium-specific deletion of full β -catenin function causes the arrest of tooth development at E14.5 in the mouse (11). To be able to distinguish between the role of β -catenin as transcriptional regulator, and as key component of the adherens junctions, we exploited the recently generated β -catenin double mutant allele (dm) (3). This allele encodes for a transcriptionally non-functional protein, which fully retains its structural contribution to the adherens junctions (3). We show that K14-Cre; *Ctnnb1*^{flox/dm} mice (K14- β -cat-dm) exhibit arrested incisor development at E14.5 (compare Fig. 1P-R with Fig 1S-U; Fig. S2), in accordance to the previously published results (11).

We then investigated whether the β -catenin transcriptional cofactors Bcl9/9l had similar roles in early stages of odontogenesis. For this purpose, we generated mice lacking Bcl9/9l in the epithelium by combining the K14-Cre driver with LoxP-containing alleles of *Bcl9/9l* (15) (K14-Cre; *Bcl9*^{flox/flox}; *Bcl9l*^{flox/flox} mice, referred to as K14-Bcl9/9l-flox) (Fig. 2A). In contrast to the phenotype caused by loss of β -catenin-dependent transcription (K14- β -cat-dm), incisors fully developed in the *Bcl9/9l* mutant mice (Fig. S3). However, these incisors lost their reddish/yellowish pigmentation that is characteristic of wild-type mouse incisors (K14-Bcl9/9l-WT, Fig. 2B) and present a white appearance (Fig. 2C). These results suggest that Bcl9/9l affect events involved in the production and composition of the enamel.

Bcl9/9l and Pygo1/2 act independently of β -catenin to ensure proper enamel formation

Enamel is the highest mineralized tissue in vertebrates, and is characterized by a tightly organized structure, which is necessary for its exceptional hardness and function. We applied scanning electron microscopy (SEM) to investigate possible defects in the structural organization of the enamel in *Bcl9/9l* mutant mice. Mutant teeth show a disorganized structure of enamel, notably in the orientation, arrangement, and thickness of the enamel rods (Compare Fig. 2E, F to 2G, H). The white and glossy appearance of the incisors in K14-Bcl9/9l-flox mutants additionally suggests lack of iron, an element that increases the hardness of enamel in rodents (16). We used energy-dispersive X-ray spectroscopy (EDS) to quantify the relative abundance of iron and other enamel-composing elements such as magnesium (Mg^{2+}) and calcium (Ca^{2+}). EDS showed that in wild-type incisors the iron is enriched within the enamel surface and accounts for approximately 4% of total enamel mass (Fig. 2I). In contrast, enamel of K14-Bcl9/9l-flox incisors contains less iron (0.5% of its total mass; Fig. 2J). Consistent with the observed iron loss and the important role of iron in enamel hardness, incisors of K14-Bcl9/9l-flox mutants show deterioration of the outermost enamel layer (see the enamel surface in Fig. 2F with Fig 2H). The relative abundance of all other elements was not affected in the enamel of the mutant incisors (Fig. 2I-J). Histological analysis confirmed this observation showing absence of iron pigmentation in mutant incisors (Fig. 2K-P).

To investigate the molecular mechanisms that underlie the enamel phenotype in the K14-Bcl9/9l-flox mutants, we sought to understand whether Bcl9/9l exert their role in enamel formation by interacting with their known partners, β -catenin and Pygo1/2 (2, 4, 5, 7). Therefore, we first abrogated the interaction between β -catenin and Bcl9/9l, mediated by its HD2 domain, by generating a genetic *Bcl9/9l* model in which this domain is deleted (K14-Cre; *Bcl9^{flox/ΔHD2}*; *Bcl9^{flox/ΔHD2}* - K14-Bcl9/9l-ΔHD2; Fig. 3A) (10). Teeth of K14-Bcl9/9l-ΔHD2 mice did not display any obvious enamel defect, and were indistinguishable from those of control littermates (compare Fig. 3B with Fig 3C, D). This demonstrates that Bcl9/9l function independently of β -catenin in enamel formation. We next investigated the effect of the other Bcl9/9l interactors, Pygo1/2, on amelogenesis. K14-Cre mediated deletion of *Pygo1/2* (K14-Cre; *Pygo1^{flox/flox}*; *Pygo2^{flox/flox}*, - K14-Pygo1/2-flox; Fig. 3E) clearly recapitulates the enamel defects observed in the K14-Bcl9/9l-flox mice (Fig. 3F-H). Notably, abrogating just the interaction between Bcl9/9l and Pygo1/2 proteins in K14-Cre; *Bcl9^{flox/ΔHD1}*; *Bcl9^{flox/ΔHD1}* (K14-Bcl9/9l-ΔHD1; (17)) is also sufficient to perturb enamel structure (Fig. S4).

Cytoplasmic Bcl9 interacts with ameloblast-specific proteins

The independence of Bcl9/9l from β -catenin-mediated transcription, together with the prominent cytoplasmic localization, strongly suggest that Bcl9/9l may interact with molecules localized in the cytoplasm of ameloblasts. To test this, we collected ameloblasts from incisors dissected from newborn mice, extracted total protein content, and performed immunoprecipitation experiments followed by mass spectrometry analysis (Fig. 4A, S5). The ameloblasts-specific Bcl9 interactome was significantly enriched for proteins involved in exocytosis, membrane-bound vesicles, and extracellular vesicular trafficking (Fig. 4B, Table S1; the statistical Gene-Ontology based analysis was performed with DAVID, <https://david.ncifcrf.gov>). Among the new interactors, we found Amelogenin, the main extracellular matrix protein required for enamel development and maturation. (Fig. 4B, C) (18, 19). Consistent with the Bcl9/9l-phenotype described above, Amelogenin is required for the inclusion of minerals into the growing hydroxyapatite crystals of enamel and, furthermore, for directing the organization of the enamel rods (20). Confocal immunofluorescence was performed to further analyze the possible interaction between Amelogenin and Bcl9, and revealed that Amelogenin and Bcl9 colocalize in the cytoplasm of secretory and mature ameloblasts (Fig. 4D, E), indicating an interplay between Amelogenin and Bcl9. We performed immunolabelling in K14-Bcl9/9l-flox incisors and found that although Amelogenin is still present, the pattern of its localization appears impaired (Fig. 4F), suggesting a potential role of Bcl9/9l in Amelogenin trafficking and secretion. Notably, Amelogenin protein is less abundant in K14-Bcl9/9l-flox incisors (Fig. 4F, G), whilst its mRNA is unaffected (Fig. 4H, left panel). Consistently, the mRNA of the most important genes known to regulate enamel formation is also unchanged in the mutant condition, further indicating the absence of a transcriptional regulation mediated by Bcl9/9l (Fig. 4H). Performing cytoplasmic versus nuclear protein fractionation, we confirmed the exclusive cytoplasmic localization of Bcl9, while Pygo2 is localized in both the cytoplasm and the nucleus of ameloblasts (Fig. 4I). Remarkably, Bcl9 and Pygo2 appear only to co-localize in the proximity of the most-apical surface of ameloblasts, the region where Bcl9 also co-localizes with Amelogenin (Fig. 4L), suggesting a potential role of the Bcl9-Pygo2 complex in the secretion of the enamel components. The hypothesis that cytoplasmic Bcl9 act along the secretory pathway is reinforced by further confocal microscopy studies showing that Bcl9/9l co-

localize with Calnexin and COP-I, which are specific molecules of the endoplasmic reticulum and Golgi apparatus, respectively (Fig. S6, S7).

Discussion

In the present study we investigated the role of the Wnt- β -catenin transcriptional regulators Bcl9/9l and Pygo1/2 in the continuously growing mouse incisor, a valuable model that allows the simultaneous investigation of processes involved in stem cell function and differentiation. Bcl9/9l and Pygo1/2 are both expressed in incisors, in the enamel-producing ameloblasts, where they are predominantly localized in their cytoplasm. The cytoplasmic localization is unexpected, given the role of these proteins as β -catenin transcriptional cofactors, and suggests that Bcl9/9l and Pygo1/2 exert a non-transcriptional role in enamel formation.

Previous studies have shown that the loss β -catenin in the dental epithelium causes the arrest of tooth development at E14.5 in mice (11). However, β -catenin is a dual function protein, regulating both the coordination of cell-to-cell adhesion at the adherens junctions and the Wnt- β -catenin-dependent transcription (2). Consequently, β -catenin full deletion does not allow distinguishing between the role of β -catenin either as transcriptional regulator or as key component of the adherens junctions. We therefore exploited the β -catenin double mutant, coding for a transcriptionally-inactive protein, but that fully retains its contribution to the adherens junctions (3). We show that the loss of Wnt- β -catenin-dependent transcription recapitulates the complete deletion of β -catenin, thus conclusively demonstrating that the main role of β -catenin at the onset of odontogenesis is to mediate the canonical Wnt signaling dependent transcription.

Bcl9/9l and Pygo1/2 are important Wnt- β -catenin transcriptional co-factors. In contrast to what observed upon loss of β -catenin transcription, teeth develop in mice lacking Bcl9/9l in the dental epithelium. However, incisors of adult mutant mice display severe defects in enamel, such as misplaced hydroxylapatite enamel rods, which in wild-type teeth are arranged in tightly ordered three-dimensional arrays interspersed with interrod enamel. This peculiar arrangement of rods serves to deflect and arrest cracks within enamel, thus ensuring its hardness and resistance to mechanical stresses (21). Enamel is the highest mineralized tissue of the mammalian body. It is mainly composed of minerals (97%), water, residual organic molecules, and small amounts of substituting ions that strongly affect its properties (21). In rodent incisors in particular, iron (Fe^{2+}) deposition is linked to the yellow pigmentation of the outermost layer of enamel and strongly increases its hardness (16). Moreover, iron provides additional resistance to bacterial toxins and acids that prevent pathological tooth alterations such as caries (16). Bcl9/9l mutant enamel is characterized by the nearly complete absence of iron. The combination of rods disorganization and iron deficiency in the enamel of *Bcl9/9l* mutant mice leads to a severe reduction of its resistance, resulting in frequent incisor fractures.

The function of Bcl9/9l in enamel formation is independent of their interaction with β -catenin, as shown by deletion of the HD2 domain, which allows the interaction between β -catenin and Bcl9/9l (10). In contrast, *Pygo1/2* deletion clearly recapitulates the enamel defects observed in the Bcl9/9l mutant mice, indicating that Pygo1/2 are important partners of Bcl9/9l for the formation of this structure. Remarkably, the sole abrogation of the interaction between Bcl9/9l and Pygo1/2 proteins is sufficient to induce defective enamel formation. We thus conclude that Bcl9/9l and Pygo1/2 act together as a functional unit to ensure proper

amelogenesis, independently of β -catenin-dependent transcription. Collectively, our data indicate that Bcl9/9l and Pygo1/2 have fundamental roles in late tooth developmental processes such as enamel formation and maturation, clearly in contrast with the early effects of β -catenin in odontogenesis.

The independence of Bcl9/9l from β -catenin-mediated transcription, together with their cytoplasmic localization in ameloblasts, prompted us to test whether Bcl9/9l interact with other cytoplasmic molecules to exert their specific functions. Indeed, we found that Bcl9 binds with several proteins involved in exocytosis, membrane-bound vesicles, and extracellular vesicular trafficking. Surprisingly, Amelogenin, which is the main extracellular matrix protein required for enamel development and maturation (18, 19), is among the Bcl9 cytoplasmic interactors that we detected. The importance of this molecule in enamel formation has been highlighted in human pathologies affecting teeth, where mutations in *AMELX*, the gene encoding for Amelogenin, are responsible for severe enamel defects, regrouped under the term Amelogenesis Imperfecta (22). Of note, the enamel phenotype observed upon mutations in *Bcl9/9l* closely resembles the one previously observed in mice where the N-terminus of Amelogenin was mutated (23, 24). We speculate that Amelogenin functions rely on its interaction with Bcl9/9l, and perhaps this interaction is mediated by the N-terminus domain of Amelogenin. In the incisors of Bcl9/9l mutant mice, both Amelogenin localization and quantity appear impaired when compared to control specimens. Nevertheless, the deletion of Bcl9/9l does not lead to significant alterations in the expression of genes coding for Amelogenin and other important genes encoding for proteins important for enamel formation, such as Ameloblastin, Enamelin, Kallikrein-4, and MMP20. This is additional evidence indicates a non-transcriptional role of Bcl9/9l in amelogenesis, and it is further reinforced by (i) the absence of Bcl9 in the nucleus, (ii) its co-localization with Pygo2 at the apical, secretory portion of ameloblasts, as well as (iii) with molecules specific for the Golgi apparatus and endoplasmic reticulum. Although we cannot exclude that Bcl9 and Pygo2 may still retain some nuclear function, we can conclusively show that they do not participate in the β -catenin dependent transcription during early odontogenesis. At more advanced developmental stages, the Bcl9-Pygo2 complex regulates enamel fine-tuning by direct physical interaction with ameloblast specific proteins without regulating their transcription. It will constitute an interesting line of experimentation to investigate the role of Bcl9/9l and Pygo1/2 in other cell-types that produce mineralized extracellular matrices, such as the odontoblasts and osteoblasts.

In an increasing number of contexts in which Bcl9/9l and Pygo1/2 are implicated as transcriptional regulators of Wnt signaling (2, 25, 26), our work provides an unexpected, alternative viewpoint, in which Bcl9/9l and Pygo1/2 can act in the cytoplasm, independently of β -catenin, and are key determinants to regulate precise and refined developmental processes. This implies that transcriptional regulators of the highly conserved Wnt signaling pathway can fine-tune late developmental events via a previously undetected cytoplasmic activity (Fig. 4K). We propose that Bcl9/9l and Pygo1/2 might exert this function by influencing the trafficking or the stability of enamel proteins and iron deposition through a not yet identified mechanism. This new role could represent an exaptation that appeared in association with highly specialized structures such as enamel, a tissue present uniquely in vertebrates (27, 28). On the other hand, these functions might constitute more general features of Bcl9/9l and Pygo1/2 that have so far been completely overlooked.

Despite their clinical relevance, the molecular mechanisms responsible for variability in tooth vulnerability observed in humans are essentially unknown. The finely tuned structure of the enamel is of crucial importance for its ability to resist insults, such as bacterial toxins, acids, and mechanical stresses that can lead to a variety of dental pathologies. Bcl9/9l and Pygo1/2 are fundamental to maintaining the structural integrity of enamel. It is tantalizing to hypothesize that genetic variations or mutations in their genes might influence the predisposition to dental defects related to enamel fragility in humans. Thus, as hitherto unrecognized players in the fine-tuning of the enamel structure and composition, Bcl9/9l and Pygo1/2 may contribute to the differential susceptibility to dental pathologies in humans.

MATERIAL AND METHODS

Animals and tissue processing

All mice were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich.

The mutant animals used in this study were generated by crossing mutant alleles in *Ctnnb1*, *Bcl9/9l* and *Pygo1/2* with a *K14-Cre* recombinase driver. For the generation of the conditional *Bcl9/9l* alleles see Deka *et al.*, 2010 (15), and the *Pygo1/2* conditional alleles, Cantù *et al.*, 2013 (17). The *Bcl9/9l* deletion mutants were generated by Cantù *et al.*, 2014 (10). The β -catenin dm allele (transcriptionally null, “double mutant”) was generated by Valenta *et al.*, 2011 (3).

In order to facilitate the complete recombination of the alleles, “K14-Bcl9/9l-flox” mice were generated by crossing the LoxP-containing alleles (K14-Cre; *Bcl9*^{flox/flox} *Bcl9*^{flox/flox}) with constitutive null alleles (*Bcl9*^{flox/KO} *Bcl9l*^{flox/KO}) in K14-Cre; *Bcl9*^{flox/KO}; *Bcl9l*^{flox/KO} mice.

Embryonic age was determined according to the vaginal plug (day 0) and confirmed by morphological criteria. Pregnant animals were sacrificed by cervical dislocation and the embryos were surgically removed into Dulbecco’s phosphate-buffered saline (PBS), pH 7.4. Dissected heads from the embryonic day 14.5 (E14.5) and new-born pups (P0) to were fixed in 4% paraformaldehyde (PFA) in PBS for 24 hours at 4°C.

Adult animals were anesthetized with ketamine/xylazine and perfused with 4% PFA. Heads were then post-fixed for up to 7 days in 4% PFA. For immunohistochemical analysis, fixed heads were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated and embedded in paraffin.

Immunohistochemistry

Immunohistochemistry was performed on 5-10 μ m thick paraffin sections. Briefly, endogenous peroxidases were quenched in a solution of 0.3% H₂O₂ in methanol and heat-induced antigen retrieval in 10 mM trisodium citrate buffer, pH 6.0, was performed. For the staining procedure, the Vectastain ABC Kit was used (ABC Kit, Vector Laboratories, Servion, Switzerland). As chromogenic substrate, SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma-Aldrich, D4293) were used. Omission of the primary antibody served as negative control. Stained sections were counterstained with toluidine blue and mounted with Glycergel (C0563, Dako, Dako North America Inc.). Pictures were taken using the Leica DFC420C camera and the Leica Application Suite (LAS) software.

The following primary antibodies were used: anti-BCL9 (Abcam ab54833 or ab37305); anti-BCL9l (Abnova PAB19408) anti-Pygo2 (Novus Biologicals NBP1-46171); anti- β -catenin

(BD Clone 14, 610154); anti-Amelogenin X (Abcam ab153915); anti-COP-I (Abcam ab6323); anti-Calnexin (Abcam ab31290).

Immunofluorescence

Immunofluorescence was performed on 5-10 μm thick paraffin sections. Heat-induced antigen retrieval in 10mM trisodium citrate buffer, pH 6.0, was performed, followed by blocking in a solution of PBS + 2% normal goat/donkey serum + 0.5% Tween-20. The sections were then incubated with primary antibodies (see above) dissolved in blocking solution overnight at 4°C. After extensive washing with PBS, the sections were then incubated with a fluorescently labeled secondary antibody (Alexa 488 goat anti-mouse, Alexa 555 goat anti-rabbit, 1:500). Nuclei were stained with DAPI (1:1000, Sigma-Aldrich Chemie, Germany). Slides were mounted with with FluorSave Reagent (Merck Millipore, 345789), and imaged with a Zeiss LSM 710 confocal microscope.

Western blot

Total protein extracts were prepared according to standard protocols. Cytoplasmic versus nuclear protein fractionation was performed as previously described (17), and proteins were subjected to SDS-PAGE separation and blotting. The following antibodies were used: anti-Pygo2 (Novus Biological, NBP1-46171, and Santa Cruz, sc-74878); anti-BCL9 (Abcam ab54833 or ab37305); anti-histone H4 (Upstate); anti-Keratin 5 (PRB-160P, BioLegends); anti-Amelogenin X (Abcam ab153915). Antibody binding was detected by using an appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (GE Healthcare).

Microcomputed tomography (MicroCT)

Adult perfused mouse heads were washed in PBS and progressively dehydrated to 70% ethanol for MicroCT analysis. The MicroCT scans were performed using a commercially available μCT unit (Specimen MicroCT 40, Scanco Medical, Brüttisellen, Switzerland) with all imaging parameters kept identical during all examinations (tube voltage, 70 kV, tube current 114 μA ; isotropic resolution, 18 μm). The original images were converted into the RAW-format using the proprietary software of the MicroCT device and imported in the 3D reconstruction program VGStudio Max (Volume Graphics, Heidelberg, Germany). All the analysed samples were segmented manually using wild type teeth as reference for the grey level values corresponding to the single mineralized tissues (enamel, dentin, bone).

Backscattered Scanning Electron Microscopy (SEM) and elemental analysis

Fully mineralized lower hemi-jaws were dissected from perfused adult (approx. 3 months of age) K14-Bcl9/9l-flox, K14-Bcl9/9l- ΔHD2 and K14-Pygo1/2-flox, and the respective Bcl9/9l-flox, Bcl9/9l- ΔHD2 and Pygo1/2-flox controls. Soft tissues were removed manually. The lower jaws were then dehydrated and embedded in Technovit 7200 VLC (Heraeus Kulzer, Wehrheim, Germany). Light-polymerized blocks were mounted on aluminium stubs, polished and coated with a 10-15 nm thick layer of carbon. Thereafter, they were examined using a Tescan EGATS5316 XMSEM (Tescan, Brno, Czech Republic) operated in BSE mode. Elemental composition of enamel was analysed with the aid of energy-dispersive X-ray spectroscopy (EDS). A Si(Li) detector (Oxford Instruments, Wiesbaden, Germany) served for recording EDS spectra using an accelerating voltage of 7 kV, a working distance of 23 mm, and a counting time

of 100 s. For the quantitative analysis of these spectra, the Inca energy software (Oxford Instruments) was used.

Immunoprecipitation and mass spectrometry

Dissected ameloblasts were minced in cold PBS, and treated with a hypotonic lysis buffer (20 mM Tris-HCl; 75 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA; 0.5% NP-40; 5% Glycerol). Protein extracts obtained were incubated with 1 µg of anti-BCL9 antibody (abcam ab54833 or ab37305) and ProteinA-conjugated Sepharose beads (GE Healthcare); they were then diluted in lysis buffer to a final volume of 1 mL. After 4 hours of incubation at 4°C on a rotating wheel, the beads were spun down, and washed three times in lysis buffer. All steps were performed on ice, and all buffers were supplemented with fresh protease inhibitors (Complete, Roche) and 1 mM PMSF.

For detecting the proteins in Western Blot, the pull down reactions were treated with “Laemmli buffer“, boiled at 95°C for 15’, and subjected to SDS–PAGE separation and blotting on PVDF membrane. The PVDF membrane was probed with the anti-Amelogenin X.

For LC-MS/MS analysis, samples were treated as in Biner et al., 2015. Briefly, protein samples were dissolved in 100 mM NH₄HCO₃ buffer, pH 8, and reduced with 5 mM DTT for 45 min at 50 °C. Reduced Cys was modified with 20 mM iodoacetamide at room temperature for 60 min in the dark. Modification was stopped by adding 10 mM DTT and incubation for 15 min. Enzymatic cleavages were performed with trypsin, trypsin/Lys-C (both Promega), and Glu-C (Roche Diagnostics). All digests were incubated overnight at 37 °C and 700 rpm and stopped by adding TFA to a final concentration of 0.5%. The samples were desalted on ZipTip C18 tips (Merck Millipore). Desalted samples were dried completely in a vacuum centrifuge and reconstituted with 20 µl of 3% ACN, 0.1% formic acid. 5-10 µL of each peptide solution was analyzed on a Q-Exactive or Fusion mass spectrometer (Thermo Scientific) coupled to an EASY-nLC1000 (Thermo Scientific). Instrument parameters are based on the “sensitive” method published by Kelstrup and colleagues, with slight modifications. Full scan MS spectra were acquired in profile mode from 300-1700 m/z with an automatic gain control target of 3e6, an Orbitrap resolution of 70’000 (at 200 m/z), and a maximum injection time of 120 ms. The 12 most intense multiply charged ($z = +2$ to $+8$) precursor ions from each full scan were selected for higher-energy collisional dissociation fragmentation with a normalized collision energy of 30 (arbitrary unit). Generated fragment ions were scanned with an Orbitrap resolution of 35’000 (at 200 m/z) an automatic gain control value of 5e4 and a maximum injection time of 120 ms. The isolation window for precursor ions was set to 2.0 m/z and the underfill ratio was at 1% (refereeing to an intensity of 4.2e3). Each fragmented precursor ion was set onto the dynamic exclusion list for 90 s. Peptide separation was achieved by RP-HPLC on an in-house packed C18 column (150 mm x 75 µm, 1.9 µm, C-18 AQ, 120 Å, Dr. Maisch GmbH, Germany). Samples were loaded with maximum speed at a pressure restriction of 400 bar and separated with a linear gradient from 3-25% solvent B (0.1% formic acid in ACN, Biosolve BV, Netherlands) in solvent A (0.1% formic acid in H₂O, Biosolve BV, Netherlands) at a flow rate of 250 nl/min. The column was washed after the separation by flushing with 95% solvent B for 10 min and automatically equilibrated prior to the next injection.

LC-MS/MS data were analyzed with Proteome Discoverer (version: 1.4.0.288; DBVersion: 79) in combination with MASCOT (version: 2.4). The search parameters were: max. missed cleavage = 3, precursor ion tolerance = 10 ppm, fragment ion tolerance = 50 mmu, fixed modification = Carboxyamidomethyl (C), variable modification = Oxidation (M)/amidation (protein C-terminus). Searches were performed using the MASCOT decoy option and results

were filtered with percolator. Only peptides having a percolator score of $q > 0.01$ were considered as trustworthy. Data analysis was performed using Scaffold – proteome software (<http://www.proteomesoftware.com/products/scaffold/>).

RNA isolation and real time PCR

Total RNA was isolated from incisors dissected from pups using the RNeasy Plus Universal Minikit (Qiagen), and further purified by ethanol precipitation. RNA retrotranscription was performed using the iScript™ Reverse Transcription Kit (Bio-Rad, Switzerland). Real-time PCRs were performed in an Illumina Eco Real-Time PCR System (Illumina), using Power Sybr Green ® (Life Technologies, Switzerland) as reaction mix. The following primers were used: Amelx (5'- CCC TAC CAC CTC ATC CTG GA-3', 5'- GAG GCT GAA GGG TGT GAC TC – 3'), Ambn (5'-AGTGAAAATGAGCCTCGCCG-3', 5'-CGTTGAGTCCCTGCAAGCTT-3'), Enam (5'-GTCTCTGCTGCCATGCCATT-3', 5'-GGAGGGTACTGTGGAGGCAT-3'), KLK4 (5'-TTGCAAACGATCTCATGCTC-3', 5'-TGAGGTGGTACACAGGGTCA-3'), MMP20 (5'-CTTTCCCCAGCTAATGTCCA-3', 5'-CTTGGGAACCCGAAGTCATA-3'). For a comparison of the groups a multiple t-test analysis was performed using GraphPad Prism v6.05 (GraphPad Software, Inc., La Jolla, CA, USA). Data were considered significant at $p < 0.05$.

List of supplementary materials:

Figure S1: Bcl9 and Pygo2 are expressed in the differentiated secretory ameloblasts but not in the stem cell niche (labial cervical loop).
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Table S1

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FIGURE LEGENDS

Fig. 1. The β -catenin cofactors Bcl9, Bcl9l and Pygo2 are expressed in the developing tooth. (A) Schematic representation of mouse incisors at embryonic day 15.5 (E15.5) and (B) of a longitudinal section of mouse lower incisor at birth (P0). dm=dental mesenchyme; de=dental epithelium; Labial (laCL) and lingual (liCL) cervical loops; dp=dental pulp (dp). (C) Schematic representation of the differentiated dental epithelium; si=stratum intermedium; en=enamel; cyt=cytoplasm; nuc=nucleus; am=ameloblast. (D-O) Immunohistochemistry for β -catenin, Bcl9, Bcl9l and Pygo2 at E14.5 and P0. Each staining shown is a representative one performed on at least of N=3 individual mice. (P) Scheme of Chain-of-adaptors complex. (Q) Transversal section of wild-type developing upper incisors at E15.5 (hematoxylin&eosin). (R) Higher magnification of control tooth germ at E15.5. When β -catenin-dependent transcription is blocked in K14-Cre-bcat-dm embryos (S), early tooth development is arrested (N=3) (T, U).

Fig. 2. Conditional loss of Bcl9/9l causes enamel defects in adult mice. (A) Bcl9/9l tether Pygopus at the β -catenin-dependent transcriptional complex. (B) Control adult incisors. (C) Disruption of the β -catenin transcriptional complex in K14-Bcl9/9l-flox mice. (D) K14-Bcl9/9l-flox incisors appear white and glassy. Please note that both upper incisors are present, but one is fractured, a phenomenon that frequently happens in mutant mice. We interpret this as an important evidence of the decreased hardness of their enamel. (E-H) Scanning electron microscope (SEM) imaging of a wild-type (E-F) and mutant (G-H) lower incisor reveals the loss of the tightly ordered disposition of the hydroxylapatite rods in the Bcl9/9l mutant enamel. (I-J) Energy-dispersive X-ray spectroscopy (EDS) shows the relative elements composition of the K14-Bcl9/9l-WT and K14-Bcl9/9l-flox enamel. 1-4 indicate the enamel regions analyzed at the tooth tip (asterisks in E and G). (L-Q) Toluidine staining of ground sections of wild-type (L-N) and Bcl9/9l mutant (O-Q) incisors – from less mature (L, O) to fully matured (tip: N, Q) enamel. All experiments have been performed with N>3 individual animals.

Fig.3. β -catenin transcription-independent, but Pygopus-dependent role of Bcl9/9l. (A) When the HD2 domain is deleted the Bcl9/9l-Pygopus complex cannot participate in the β -catenin dependent transcription. (B, C) No enamel defect follows the deletion of the HD2. (D) SEM analysis of K14-Bcl9/9l- Δ HD2 enamel. (E) K14-Cre mediated deletion of *Pygo1/2* in the oral epithelium. (F, G) K14-Pygo1/2-flox mice display a phenotype strikingly similar to the one induced by the loss of Bcl9/9l. (H) SEM analysis of K14-Pygo1/2-flox. All experiments have been performed with N>3 individual animals.

Fig. 4. Bcl9 interactome in the ameloblasts. (A) Workflow for ameloblasts dissection and Bcl9 interactome analysis (N=2). (B) The ameloblast-specific Bcl9-interactome is enriched with proteins involved in cytosolic/extracellular trafficking. (C) Validation of the Bcl9-Amelogenin interaction (N=2). IP/immunoprecipitation; WB/western blot. (D) Confocal immunofluorescence analysis of the co-staining of Bcl9 with Amelogenin (N=3 stainings performed on independent individuals). (E) Fiji-ImageJ based colocalization analysis of Bcl9 and Amelogenin. Grey indicates the areas of colocalization on a single focal plane (1 Airy Unit, AU). (F) Amelogenin localization in K14-Bcl9/9l-flox ameloblasts. (G) Western blot showing Amelogenin extracted from wild-type (WT) and K14-Bcl9/9l-flox teeth. Keratin 5 is used as loading control. Each lane represents an independent individual mouse (N=2). (H) Real Time quantitative RT-PCR

performed on isolated dental epithelia from wild-type (black bars) and mutant (red bars) teeth (N=5) to quantify mRNA abundance of genes important for enamel formation. **(I)** Cytoplasmic versus nuclear protein fractionation of ameloblasts visualized with western blot analysis (N=2). Histone H4 is used to specifically mark the nuclear fraction. **(J)** Bcl9 and Pygo2 co-staining in the ameloblasts, imaged with confocal microscopy (1 AU) (N=2). Bcl9 (green), Pygo2 (red). Note the signal overlap at the most-apical, secretory region of ameloblasts. **(K)** Bcl9/9l and Pygo are key determinants of late enamel development through a previously unrecognized cytoplasmic function.

E15.5

P0

P0 - magn.

β -catenin

Bcl9

Bcl9l

Pygo2

wild type

K14- β -catenin dm

K14-Bcl9/9L WT

I

Spectrum	O	Na	Mg	P	Cl	Ca	Fe	Sum
1	47.66	0.63	0.00	17.87	0.35	29.52	3.97	100.00
2	46.84	0.60	0.00	19.29	0.45	32.83	0.00	100.00
3	47.60	0.67	0.00	19.02	0.35	32.35	0.00	100.00
4	48.14	0.83	0.15	18.86	0.29	31.74	0.00	100.00

J

Spectrum	O	Na	Mg	P	Cl	Ca	Fe	Sum
1	47.09	0.69	0.00	19.29	0.39	32.03	0.51	100.00
2	46.71	0.68	0.00	19.30	0.39	32.93	0.00	100.00
3	46.56	0.69	0.00	19.30	0.35	33.11	0.00	100.00
4	46.76	0.74	0.16	19.24	0.32	32.78	0.00	100.00

K14-Bcl9/9I-ΔHD2

K14-Pygo1/2-flox

B

GO terms	FOLD ENRICHMENT	P-VALUE	Examples
extracellular exosome	4.25	6.98E-07	Annexin A1, Annexin A6 Lumican
extracellular vesicle	4.23	7.69E-07	
extracellular organelle	4.22	8.05E-07	
membrane-bounded vesicle	3.5	2.68E-05	
vesicle	3.22	1.23E-04	Amelogenin X
extracellular region part	3.21	5.64E-05

Supplementary Materials for

A cytoplasmic role of Wnt/ β -catenin transcriptional cofactors in tooth enamel formation

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Figs. S1 to S7
Table S1

SUPPLEMENTARY FIGURES

Fig. S1

Fig. S1: Bcl9 and Pygo2 are expressed in the differentiated secretory ameloblasts but not in the stem cell niche (labial cervical loop). Confocal images showing the expression of Bcl9 and β -catenin (A) (marking the epithelial ameloblasts), and Pygo2 and β -catenin (B). No detectable Bcl9 and Pygo2 proteins mark the labial cervical loop, which constitutes the stem cell niche. (C) No Bcl9 (upper panels) and Pygo (lower panels) staining is detectable in K14-Bcl9/9l-flox and K14-Pygo1/2-flox ameloblasts, respectively. Each staining has been performed on sections deriving from at least 3 independent animals.

Fig. S2

Fig. S2: β -catenin transcriptional output is required for early tooth development. At (A, B) Red arrows indicate upper and lower incisors' developing tooth germs. When β -catenin transcriptional function is abrogated in the oral epithelium in K14- β -cat-dm - tooth

development does not proceed beyond bud stage (mutant upper and lower incisors, in **C** and **D**, respectively); (N=3).

Fig. S3

Fig. S3. K14-Cre mediated deletion of *Bcl9/9l* does not cause early tooth development arrest. μ CT analysis of adult heads shows that, as in control mice (**A**), in K14-Bcl9/9l-flox individuals (**B**) tooth development proceeds without any obvious morphological alteration: mutant animals reach adulthood with functional teeth in laboratory conditions. Nevertheless, mutant teeth display a subtle decrease in enamel thickness (enamel appears as a bright white tissue). In addition, molar cusps are shorter and less sharp (N>3). We interpret this difference as a consequence of the enamel defect induced by the loss of cytoplasmic Bcl9/9l, described in Fig. 2 and S3.

Fig. S4

Fig. S4: The Bcl9/9l-Pygo1/2 interaction is required for proper enamel formation. (A) Bcl9/9l interact with Pygo proteins via the HD1 domain - when this domain is deleted the Bcl9/9l-Pygo1/2 cannot form (10). (B, C) K14-Bcl9/9l-ΔHD1 mice (K14-Cre; *Bcl9*^{lox/ΔHD1}; *Bcl9l*^{lox/ΔHD1}) display a phenotype consistent with to the one induced by the loss of Bcl9/9l or Pygo1/2 in the oral epithelium (N>3). (D) SEM analysis of K14-Bcl9/9l-ΔHD1 mice reveals the loss of the ordered disposition of the hydroxylapatite rods.

Fig. S5

Fig. S5: Search for ameloblasts-specific Bcl9 interactors. Protein extracts from mouse ameloblasts were subjected to pull down with an anti-Bcl9 antibody. (A) In a representative experiment we consider as potential Bcl9 interacting partners those proteins pulled-down by the anti-Bcl9 antibody (set colored in red) but not by the control preimmune serum (IgG). (B) Scatterplot showing the proteins differentially pulled down by the two antibodies. The most enriched peptides found in the anti-Bcl9 reaction expectedly map to mouse Bcl9 (red arrow); we consider this as an additional validation of the specificity of the antibody used in this study.

Fig. S6

Fig. S6: Bcl9 and Bcl9l are present in the cytoplasm along the secretory pathway. Confocal immunofluorescence analysis of the co-staining of Bcl9 (red) with Calnexin (green in **A** and **B**) or with COP-I (green in **C** and **D**). (**B**, **D**) Higher magnification of the regions indicated with a white rectangle in **A** and **C**, respectively. As for Bcl9, also Bcl9l is found along the domain of expression of the two vesicular markers Calnexin (**E**, **F**) and COP-I (**G**, **H**). Note the presence of green staining within the enamel, suggesting the possibility that Bcl9l is secreted into the extracellular matrix. Each staining has been performed on sections deriving from at least 3 independent animals.

Fig. S7

Fig. S7: Bcl9/9l colocalize with the secretory pathway proteins Calnexin and COP-I. Fiji-ImageJ based colocalization analyses of the confocal immunofluorescence images showing the co-staining of Bcl9 (green) with (A) Calnexin (red), (C) COP-I (red), or (B, D) DAPI (red); Bcl9l (green) with (E) Calnexin (red) and (F) COP-I (red). Grey represents the regions of signal colocalization. Note in B and D the absence of overlapping signal between Bcl9 molecules and DAPI, confirming that Bcl9 is prevalently cytoplasmatic. The sections were imaged with a Zeiss lsm 710 confocal microscope, using a pinhole size of 1

airy unit (AU) to capture emission wavelength deriving from a single focal plane. Each staining has been performed on sections deriving from at least 3 independent animals.

Table S1

Accession	Protein Name
E9Q7G0_MOUSE	Protein Numa1 OS=Mus musculus GN=Numa1 PE=1 SV=1
PPBT_MOUSE	Alkaline phosphatase, tissue-nonspecific isozyme OS=Mus musculus GN=Alpl PE=1 SV=2
HNRL2_MOUSE	Heterogeneous nuclear ribonucleoprotein U-like protein 2 OS=Mus musculus GN=Hnrnpul2 PE=1 SV=2
FBRL_MOUSE	rRNA 2'-O-methyltransferase fibrillarin OS=Mus musculus GN=Fbl PE=2 SV=2
RMXL1_MOUSE	RNA binding motif protein, X-linked-like-1 OS=Mus musculus GN=Rbmxl1 PE=1 SV=1
SFPQ_MOUSE	Splicing factor, proline- and glutamine-rich OS=Mus musculus GN=Sfpq PE=1 SV=1
FUBP2_MOUSE	Far upstream element-binding protein 2 OS=Mus musculus GN=Khsrp PE=1 SV=2
Q8VHM5_MOUSE	Heterogeneous nuclear ribonucleoprotein R OS=Mus musculus GN=Hnrnpr PE=1 SV=1
H2B3B_MOUSE	Histone H2B type 3-B OS=Mus musculus GN=Hist3h2bb PE=1 SV=3
U5S1_MOUSE	116 kDa U5 small nuclear ribonucleoprotein component OS=Mus musculus GN=Eftud2 PE=2 SV=1
FUS_MOUSE	RNA-binding protein FUS OS=Mus musculus GN=Fus PE=2 SV=1
Q9CQM8_MOUSE	60S ribosomal protein L21 OS=Mus musculus GN=Rpl21 PE=2 SV=1
ECHB_MOUSE	Trifunctional enzyme subunit beta, mitochondrial OS=Mus musculus GN=Hadhb PE=1 SV=1
TPM3_MOUSE	Isoform 2 of Tropomyosin alpha-3 chain OS=Mus musculus GN=Tpm3
RS7_MOUSE	40S ribosomal protein S7 OS=Mus musculus GN=Rps7 PE=2 SV=1
RL9_MOUSE	60S ribosomal protein L9 OS=Mus musculus GN=Rpl9 PE=1 SV=2
SND1_MOUSE	Staphylococcal nuclease domain-containing protein 1 OS=Mus musculus GN=Snd1 PE=1 SV=1
RPN1_MOUSE	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Mus musculus GN=Rpn1 PE=1 SV=1
LUM_MOUSE	Lumican OS=Mus musculus GN=Lum PE=1 SV=2
PRDX4_MOUSE	Peroxiredoxin-4 OS=Mus musculus GN=Prdx4 PE=1 SV=1
PPIA_MOUSE	Peptidyl-prolyl cis-trans isomerase A OS=Mus musculus GN=Ppia PE=1 SV=2
ANXA1_MOUSE	Annexin A1 OS=Mus musculus GN=Anxa1 PE=1 SV=2
BAF_MOUSE	Barrier-to-autointegration factor OS=Mus musculus GN=Banf1 PE=1 SV=1
P63_MOUSE	Tumor protein 63 OS=Mus musculus GN=Tp63 PE=1 SV=1
B1AWE0_MOUSE	Clathrin light chain A OS=Mus musculus GN=Clta PE=1 SV=1
MPCP_MOUSE	Phosphate carrier protein, mitochondrial OS=Mus musculus GN=Slc25a3 PE=1 SV=1
ANXA6_MOUSE	Annexin A6 OS=Mus musculus GN=Anxa6 PE=1 SV=3
PCBP1_MOUSE	Poly(rC)-binding protein 1 OS=Mus musculus GN=Pcbp1 PE=1 SV=1
RL27A_MOUSE	60S ribosomal protein L27a OS=Mus musculus GN=Rpl27a PE=2 SV=5
RL5_MOUSE	60S ribosomal protein L5 OS=Mus musculus GN=Rpl5 PE=1 SV=3
P5CR2_MOUSE	Pyrroline-5-carboxylate reductase 2 OS=Mus musculus GN=Pycr2 PE=2 SV=1
O88375_MOUSE	Keratin associated protein 13 OS=Mus musculus GN=Krtap13 PE=2 SV=1
GSTP1_MOUSE	Glutathione S-transferase P 1 OS=Mus musculus GN=Gstp1 PE=1 SV=2
HUTH_MOUSE	Histidine ammonia-lyase OS=Mus musculus GN=Hal PE=1 SV=1
SMD1_MOUSE	Small nuclear ribonucleoprotein Sm D1 OS=Mus musculus GN=Snrpd1 PE=2 SV=1
PITX2_MOUSE	Isoform Ptx2C of Pituitary homeobox 2 OS=Mus musculus GN=Pitx2
GSDMA_MOUSE	Gasdermin-A OS=Mus musculus GN=Gsdma PE=2 SV=1
TXND5_MOUSE	Thioredoxin domain-containing protein 5 OS=Mus musculus GN=Txndc5 PE=1 SV=2
NUCKS_MOUSE	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 OS=Mus musculus GN=Nucks1 PE=1 SV=1
A6ZI44_MOUSE	Fructose-bisphosphate aldolase OS=Mus musculus GN=Aldoa PE=2 SV=1
ENOA_MOUSE	Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3
K2C72_MOUSE	Keratin, type II cytoskeletal 72 OS=Mus musculus GN=Krt72 PE=3 SV=1
PFKAM_MOUSE	ATP-dependent 6-phosphofructokinase, muscle type OS=Mus musculus GN=Pfkm PE=1 SV=3

TPM2_MOUSE	Isoform 2 of Tropomyosin beta chain OS=Mus musculus GN=Tpm2
A2AIM4_MOUSE	Tropomyosin beta chain OS=Mus musculus GN=Tpm2 PE=3 SV=1
E9Q450_MOUSE	Tropomyosin alpha-1 chain OS=Mus musculus GN=Tpm1 PE=3 SV=1
D3Z724_MOUSE	Protein Gm5965 OS=Mus musculus GN=Gm5965 PE=4 SV=1
MYH4_MOUSE	Myosin-4 OS=Mus musculus GN=Myh4 PE=1 SV=1
Q9D6T8_MOUSE	MCG128973 OS=Mus musculus GN=2310057N15Rik PE=2 SV=1
TERA_MOUSE	Transitional endoplasmic reticulum ATPase OS=Mus musculus GN=Vcp PE=1 SV=4
PRDX1_MOUSE	Peroxiredoxin-1 OS=Mus musculus GN=Prdx1 PE=1 SV=1
AMELX_MOUSE	Amelogenin, X isoform OS=Mus musculus GN=Amelx PE=2 SV=1
PRDX1_MOUSE	Peroxiredoxin-1 OS=Mus musculus GN=Prdx1 PE=1 SV=1
TXND5_MOUSE	Thioredoxin domain-containing protein 5 OS=Mus musculus GN=Txndc5 PE=1 SV=2
A6ZI44_MOUSE	Fructose-bisphosphate aldolase OS=Mus musculus GN=Aldoa PE=2 SV=1
ENOA_MOUSE	Alpha-enolase OS=Mus musculus GN=Eno1 PE=1 SV=3
PFKAM_MOUSE	ATP-dependent 6-phosphofructokinase, muscle type OS=Mus musculus GN=Pfkm PE=1 SV=3

Table S1: list of proteins found in two independent pull-down experiments using the anti-Bcl9 antibody, by subtracting the proteins retrieved in the control reaction (IgG).