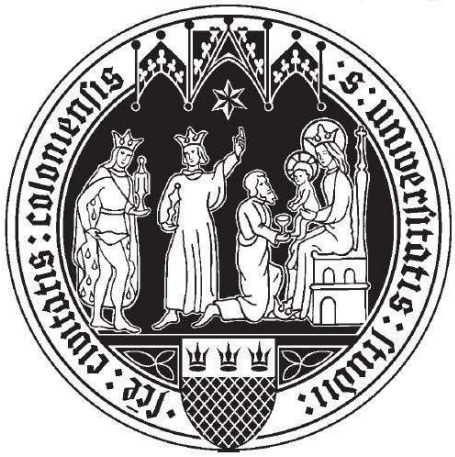
p53 and TAp63 promote terminal keratinocyte differentiation in breeding tubercles of the zebrafish



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p53 and TAp63 promote terminal keratinocyte differentiation in breeding tubercles of the zebrafish

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Abstract

p63, a member of the p53 family of transcription factors, is crucial for vertebrate epidermal development. However, different isoforms of p63 are supposed to have different if not opposing functions. There is compelling genetic evidence that ΔNp63 isoforms are needed to enhance keratinocyte proliferation and stemness. However, the role of TAp63 isoforms is not fully understood, and TAp63 knockout mice display normal epidermal development. In this thesis, we describe the epidermal defects of zebrafish mutants specifically lacking TAp63 isoforms. TAp63 as well as p53 mutant zebrafish present with compromised development of breeding tubercles, epidermal appendages with more advanced stratification and keratinocyte differentiation than in regular epidermis, including continuous desquamation and renewal of superficial cells by derivatives of basal keratinocytes. Additionally, those defects are further enhanced in TAp63/p53 double mutants. Furthermore, molecular analyses, treatments with chemical inhibitors and epistasis studies indicate the existence of a linear TAp63/p53 → Notch → Caspase 3 pathway required for enhanced proliferation of keratinocytes at the base of the tubercles and their subsequent differentiation in the absence of complete cell death. In summary, these studies identify the zebrafish breeding tubercles as specific epidermal structures sharing crucial features with cornified mammalian epidermis. In addition, they demonstrate essential and partially redundant roles of TAp63 and p53 to promote keratinocyte proliferation and their terminal differentiation, involving a pathway that might be conserved between fish and mammals.

Zusammenfassung

p63, ein Mitglied der p53-Familie von Transkriptionsfaktoren wird essentiell benötigt während der Entwicklung der Epidermis von Vertebraten. Andererseits scheinen verschiedene Isoformen von p63 unterschiedliche, wenn nicht sogar gegensätzliche Funktionen zu haben. Es existieren verschiedene Hinweise, dass ΔNp63 benötigt wird um Proliferation und Stammzell-Eigenschaften in Keratinozyten anzuregen. Die Funktion der TAp63-Isoform ist dagegen nur wenig verstanden, wobei TAp63-defiziente Maus-Mutanten einen eher normalen epidermalen Phänotyp zeigen. In dieser Arbeit beschreiben wir die epidermale Defekte einer Zebrafisch-Mutante, in der spezifisch die TAp63-Isoformen von p63 fehlen. TAp63-, genauso wie p53-Mutanten zeigen eine gestörte Bildung von „Breeding Tubercles", epidermalen Anhangsgebilden mit weitergehender Stratifizierung und Differenzierung verglichen mit regulärer Epidermis von Fischen, einschliesslich kontinuierlicher Schuppung und Erneuerung von äusseren Zelllagen durch Nachkommen basaler Zellen. Weiterhin sind diese Defekte noch verstärkt in TAp63/p53 Doppelmutanten.

Durchgeführte molekulare Analysen, Behandlung mit chemischen Inhibitoren und Epistase-Studien deuten auf die Existenz eines linearen TAp63/p53→ Notch → Caspase 3 Signalübertragungsweges hin, der sowohl für Proliferation an der Basis der Tuberkel, als auch für die nachfolgende terminale Differenzierung (die allerdings ohne kompletten Zelltod einhergeht) nötig ist. Insgesamt identifiziert diese Arbeit die „Breeding Tubercels" von Zebrafischen als epidermale Strukturen mit deutlichen Ähnlichkeiten zur verhornten Säugetier-Epidermis. Weiterhin werden teilweise redundante Funktionen von TAp63 und p53 sowohl in Bezug auf Proliferation als auch Differenzierung von Keratinozyten aufgezeigt die von einem molekularen Signalweg reguliert werden, der konserviert ist zwischen Fisch und Säugetier.

I. Introduction

Terminal differentiation of keratinocytes in land living animals

The epidermis is the outermost part of the integument that constitutes the boundary between the organism and the environment. Thereby, the epidermis has to protect against various physical, chemical and microbiological traumata. Additionally, most important for all land-living tetrapods, the epidermis provides a tight barrier against water loss and dehydration [Fuchs & Raghavan 2002, Medawar 1953]. Histologically, the epidermis of those animals has been classified as a multilayered, stratified and squamous epithelium that originates from the embryonic ectoderm [Fuchs 2007, Fuchs & Raghavan 2002]. The epidermis also develops certain epidermal appendages, like hairs, nails or claws. The "regular" epidermis between these appendages (especially the hair producing hair follicles) is referred as interfollicular epidermis [Fuchs 2007].

The specific functions of the epidermis are achieved particularly by terminal differentiation of keratinocytes called cornification [Candi et al. 2005]. This differentiation process is a specific characteristic of mammalian epidermis but has already been present in birds, reptiles and amphibians. In the progress of evolution, a cornified epidermis depicts an important step in the adaptation to land [Wu et al. 2004 & Alibardi 2003].

Particularly in older literature, the epidermis is only described as keratinized what means it contains high amounts of the intermediate filament keratin. However, during the last years it became clear that terminal differentiation of keratinocytes includes many more cellular hallmarks than only keratinization. All these processes together are now described as cornification. However, keratinisation and cornification are still often used as synonymous terms [Candi et al. 2005].

In mammalian - including human - epidermis different layers can be distinguished by the status of differentiation of the keratinocytes. Terminal differentiation is accompanied by differential gene expression of certain marker genes only in specific layers. Of certain importance is the so called "keratin-code" in the epidermis: Different layers are characterized by the expression of distinct keratins [Candi et al. 2005, Fuchs & Raghavan 2002]. The anatomy of mammalian epidermis together with the expression domain of certain marker genes is summarized in **figure 1**.

Proliferation occurs only in the basal layer which consists of stem cells (with unlimited capacity of cell division) and transient amplifying (TA-) cells (with limited capacity of cell divisions) [Mack et al. 2005]. The proliferation rate is precisely balanced with a desquamation process of the cornified layer at the outer surface of the epidermis. Via this epidermal homeostasis process, the epidermis is constantly rejuvenated [Candi et al. 2005].

Basal keratinocytes are attached to the underlying extracellular matrix (ECM) and the basement membrane via integrins, which mediate not only attachment, but also regulate proliferation or terminal differentiation in keratinocytes [Lippens et al. 2005]. Two different cell-ECM complexes mediate the connection between the basal cells and the basement membrane: hemidesmosomes, which connect intracellularly to the keratin filaments and focal adhesion contacts, which connect to the actin cytoskeleton. Both types of contacts contain integrin dimers [Fuchs & Raghavan 2002].

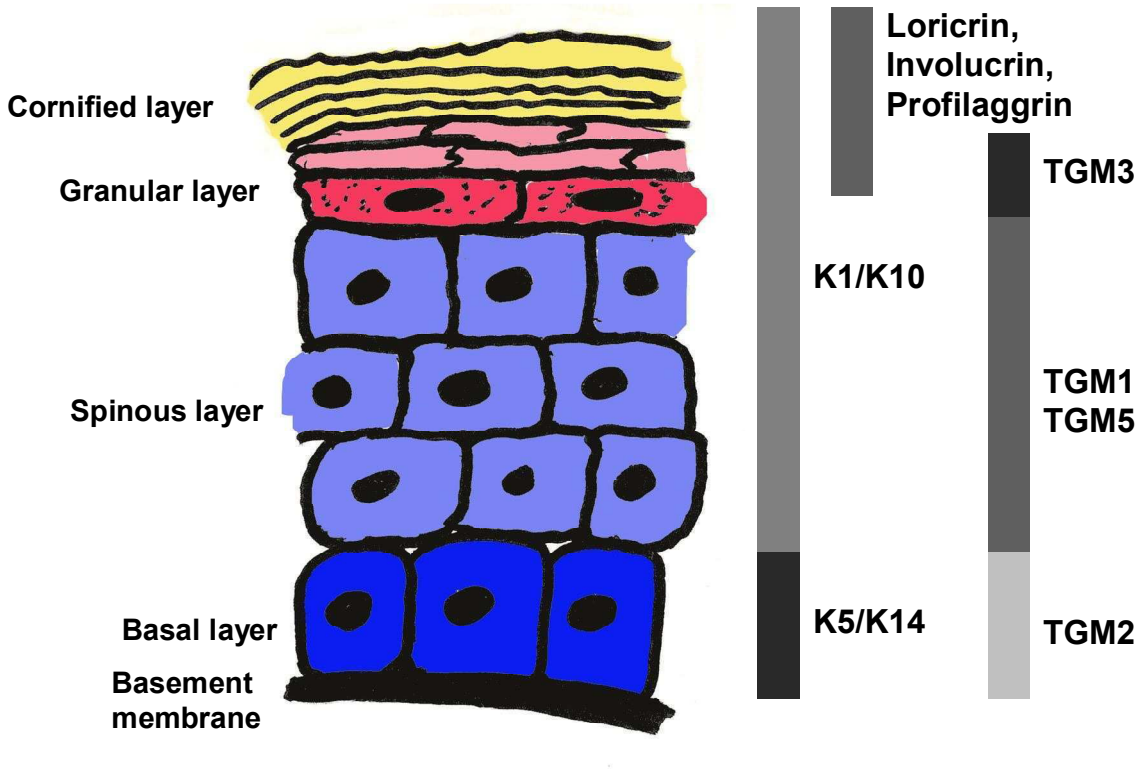


Figure 1: Anatomy of mammalian epidermis. The epidermis of mammals can be divided into basal layer, spinous layer, granular layer and cornified layer. The cells above the granular layer are dead and enucleated. Cells in the epidermis differentiate while they migrate from the basal layer to the cornified layer. Certain marker genes are expressed only in specific layers (K Keratin, TGM Transglutaminase).

Differentiation of keratinocytes starts after the cells have undergone cell cycle arrest and detached from the basement membrane [Fuchs 1995, Lippens et al. 2005]. At the molecular level, the process is initiated by unoccupied β1-integrin receptors. However, the same mechanism can also induce apoptosis or anoikis in cell culture. So far, both processes are supposed to be interdependent. The initial molecular trigger for detachment of keratinocytes and the molecular survival mechanism that prevents immediate cell death are still unidentified [Lippens et al. 2005, Levy et al. 2000, Mitra et al. 1997]. However, Notch-signalling and its target gene Hes1 are considered to play a pivotal role at the basal-suprabasal juncture to down-regulate basal genes (like K5/K14) and up-regulate spinous markers (like K1/K10) [Blanpain et al. 2006].

After detachment and initiation of terminal differentiation, keratinocytes leave the basal layer and migrate into the suprabasal compartment [Fuchs & Raghavan 2002]. This switch towards differentiation can also be seen in the transcriptional profile of the cells. While proliferating keratinocytes express keratin 5 and 14 (K5 & K14) as main intermediate filaments (bundled into KIFs = keratin intermediate filaments) of the cytoskeleton [Fuchs & Raghavan 2002, Byrne et al. 1994], differentiating cells immediately start expressing keratins 1 and 10 (K1 & K10), which replace the old K5/K14 filament network (**figure 1**) [Candi et al. 2005, Fuchs & Raghavan 2002]. This switch in keratin gene expression indicates the commitment of the epidermal cell to terminal differentiation

[Fuchs 2007]. Starting in the spinous layer, K1/K10 are expressed up to the outer cornified layer [Fuchs & Raghavan 2002, Candi et al. 2005]. The new keratin network does not only remodel the cytoskeleton of the cell to facilitate cell migration, but also regulates cell growth and cell cycle arrest via binding the adaptor protein 14-3-3σ and activating the AKT/mTOR (mammalian target of rapamycin) pathway [Kellner & Coulombe 2009, Fuchs 2007, Kim et al. 2006, Watt 2002].

In the following two to four weeks, the keratinocytes migrate through all epidermal layers to the outer surface while they differentiate into dead and enucleated corneocytes [Lippens et al. 2005, Fuchs 1995]. The cornification process includes rearrangements of the cytoskeleton, formation of a cornified envelope (CE) underneath the plasma membrane and an outer lipid envelope in the extracellular space. Keratins become the main component of the cytoskeleton and form a dense intracellular network while they are bundled into large tonofibrils (keratinization) [Fuchs 2007, Candi 2005, Matoltsy 1976]. The process ends with a flattened, dead and enucleated cell at the outer surface that has lost all its organelles and is metabolically inactive. To highlight that the differentiation process is fulfilled, these cells are thus described as corneocytes [Fuchs & Raghavan 2002].

Through all layers the cells are attached to each other by cell-cell contacts. Adherens junctions connect two cells with the intracellular actin cytoskeleton and are found particularly in the lower layers of the epidermis, whereas desmosomes are in contact with the keratin intermediate filament system and can be found in all layers [Fuchs & Raghavan 2002].

Formation of the cornified envelope starts in the granular layer [Fuchs & Raghavan 2002]. Here cells acquire electron-dense keratohyalin granules which contain profilaggrin, the precursor of filaggrin that bundles keratin intermediate filaments into large macrofibrillar cables (= tonofibrils) [Fuchs 2007] and thereby promotes the collapse of the cell into a flattened shape, the characteristic of corneocytes [Fuchs & Raghavan 2002]. Keratin intermediate filaments and filaggrin together constitute between 80 and 90 % of the total cellular protein mass in the granular layer. Both proteins are heavily covalently cross-linked and build up a platform for later reinforcement and maturation of the cornified envelope [Fuchs & Raghavan 2002]. Already in the spinous layer, specific modifications at the inner side of the desmosomes had occurred that are now reinforced in the granular layer. CE-proteins like small proline-rich proteins (SPRPs), Loricrin, Involucrin and Filaggrin, which are rich in glutamine and lysine residues [Fuchs 2007] are crosslinked to the inner side of the plasma membrane and to the keratin intermediate filament system. This crosslinking occurs all over the inner side of the plasma membrane but particularly at the desmosomes [Candi 2005, Fuchs & Raghavan 2002]. The characteristic resistance and insolubility of the cornified envelope is mainly achieved by very stable covalent isopeptide bonds, whose formation is catalyzed by Ca^{2+} dependent transglutaminases (TGMs) [Fuchs & Raghavan 2002]. Most important substrates for transglutaminases are type II-Keratins, Involucrin, Loricrin, Filaggrin, S100-Proteins and small proline-rich proteins (SPRPs) [Fuchs & Raghavan 2002]. Due to their main function, attaching the cornified envelope to keratin filaments, all these proteins are described as intermediate filament associated proteins (IFAPs) [Fuchs 1995]. Interestingly, most (but not all) of these CE proteins are arranged in mammalian genomes in one large gene cluster called

epidermal differentiation complex EDC (for example EDC in humans is a two Mb cluster with 45 genes on Chromosome 1q21) [Hoffjan & Stemmler 2007].

In the granular layer, the cells are tightly attached to each other by tight junctions. In cornifying epidermis, tight junctions are present only in this layer and play a crucial role in preventing transepidermal water loss [Brandner 2009, Mirota & Miyachi 2003, Tsuruta et al. 2002].

The cells of the outermost cornified layer are still tightly attached to each other. This is achieved by so-called corneodesmosomes, a modified desmosomal structure in which specific desmosomal proteins are substituted. Profound changes in desmosome morphology can be detected at the transition between the granular and the cornified layer. At the ultrastructural level, the cytoplasmic plaques of the desmosomes are integrated into the cornified envelope and a homogenous electron-dense plaque occurs in the intercellular space [Fuchs & Raghavan 2002]. This plaque is considered to consist of the transmembrane proteins Desmoglein and Desmocollin, members of the cadherin-family, and the extracellular protein Corneodesmosin, which is produced by the keratinocytes of the granular layer [Ishida-Yamamoto et al. 2011].

Additionally to the proteinacous cornified envelope, an extracellular lipid envelope is formed also starting at the granular layer. Lamellar bodies (= Odland bodies), which store complex series of lipids (e. g. ceramids, cholesterol esters or free fatty acids) originate from the Golgi apparatus and fuse with the plasma membrane to release their content into the extracellular space [Candi et al. 2005, Fuchs & Raghavan 2002]. Outside the cell, the lipids are directly bound to the outer cell membrane to reinforce the barrier function, or aligned into extracellular lipid lamellae to prevent water loss. Together with the lipid lamellae precursors, acidic hydrolases are extruded into the intercellular space, which are responsible for changes of the lipid composition and subsequently lipid lamellae formation. At the end of this process in the upper cornified layers, the cornified envelope is embedded in a lipid envelope and the lipids are organized as stacked lamellar sheets that occupy all intercellular space between the corneocytes. An important but still unresolved field of research are interactions between the lipid envelope and the cornified envelope [Lippens et al. 2005, Fuchs & Raghavan 2002].

Simultaneously to the formation of the cornified and the lipid envelope, degradation of the DNA and cell organelles occurs. In contrast to apoptosis, no chromatin condensation or DNA laddering occurs during cornification. Finally the nucleus disappears and no chromatin remnants remain. So far, neither any DNAse responsible for this degradation process nor the processors of nuclear destruction could be identified. [Lippens et al. 2005].

Above the granular layer, the skin is comprised only of dead cells. The fully differentiated squames resemble empty "protein sacs" consisting nearly exclusively of keratins and cross-linked CE-proteins. During the final shedding process in the uppermost layers of the *stratum corneum*, proteolytic degradation of cell-cell contacts allows squammation [Lippens et al. 2005, Fuchs & Raghavan 2002]. Especially during the later stages, cornification highly depends on specific proteases. Many CE protein precursors need proteolytic activation. Degradation of the nucleus and the organelles depends on proteases and finally degradation of cell-cell contacts during sloughing of cornified squames [Fuchs & Raghavan 2002].

As already mentioned, proliferation and squammation are tightly linked and regulated processes in epidermal maintenance [Lippens et al. 2005, Fuchs 1995]. This may be achieved by organizing the epidermis into epidermal proliferating units (EPUs), columns of hexagonally packed cells as has been shown by lineage tracing experiments. All cells of an EPU originate from single basal stem cells that occasionally divide to produce cells with more restricted proliferating capacity (TA-cells), whereas all other cells differentiate [Fuchs 2007].

Cornification is the most abundant form of cell death in mammalian epidermis. Despite many similarities (e. g. loss of the nucleus and cell organelles and activation of specific proteases including effector-caspases), cornification is clearly different from apoptosis [Lippens et al. 2005, Candi et al. 2005, Fuchs & Raghavan 2002]. Apoptosis in the skin mainly occurs as a response to cell damage by UV light in proliferating basal keratinocytes, whereas cornification is a physiological process of terminal differentiation in the suprabasal layers [Lippens et al. 2005]. Characteristically, apoptosis occurs in single cells, which thereby loose their cell-cell contacts and are phagocytized afterwards by macrophages. In contrast, cornification is associated with sheet formation (cell-cell contacts remain intact) and stratification of the epidermis, meaning that the cellular changes during the differentiation process are not restricted to single cells, but occur simultaneously in all keratinocytes of the respective epithelial sheet [Lippens et al. 2005].
Interestingly, nearly all keratins as major components of differentiated keratinocytes contain conserved caspase cleavage sites. However, keratins are not considered to be cleaved during terminal differentiation. Whether they are indeed substrates of effector-caspases during apoptosis remains still unclear [Lippens et al. 2005]. The most important features of both types of cell death are summarized in **table 1**.

In a similar way, cornification as described above occurs in all amniotes (mammals, reptiles & birds). For all these land-living animals protection from dehydration is crucial for survival [Alibardi 2002].
Reptiles resemble the primary amniotes from which birds and mammals derived. However, the mechanism of keratinization and cornification in reptiles is only poorly understood. In general, two different processes, α- and β-keratinization can be distinguished which involve α- and β-keratins. In α-keratinized layers of the epidermis, cross-reactivity with antibodies against the CE-proteins Filaggrin and Loricrin could be detected in keratohyalin-like granules (KHLG) in the granular layer. A cornified envelope is also formed in the *stratum corneum* [Alibardi 2003]. These results support the hypothesis that reptilian α-keratinization (which is softer and occurs often at locations exposed to movement) resembles mammalian keratinization, whereas β-keratinization forms the hard scales of the reptilian skin which are folds of the complete skin. β-keratins might have evolved from a single α-keratin in the course of evolution, but seem to be now an independent keratin family that does not exist in mammals at all [Alibardi & Toni 2006, Alibardi 2002]. Alternating layers of α- and β-keratinized keratinocytes facilitate shedding [Alibardi 2003]. However, not all reptiles renew the cornified tissue via a shedding process. Continuous wearing is also very common [Alibardi & Toni 2006].

Table 1: Differences between cornification and apoptotic cell death.

	Cornification	Apoptosis
General features	• Slow process (duration ca. 2 weeks) • Occurs simultaneously in all cells of an epidermal layer • Massive synthesis of specific proteins	• Fast process (48-72 hours) • Occurs in individual cells surrounded by viable neighbour cells • Limited need for protein synthesis
Cell-cell contacts	• Remain intact	• Apoptotic cells loose cell-cell contacts what causes formation of apoptotic bodies
Nuclear events	• Complete loss of nucleus • No chromatin condensation or DNA laddering • Responsible DNases unknown • Proteases that cause destruction of nuclear proteins unknown (except Desquamin)	• Incomplete loss of nucleus • Nuclear remnants detectable • Internucleosomal DNA cleavage by caspase-activated DNases (CADs) • Caspases degrade nuclear proteins (for example Lamin)
Cytoskeletal rearrangements	• Expression of different cytoskeletal components during the process • Formation of the cornified envelope and the lipid envelope • Cross-linkage between keratin filament system and cornified envelope	• Dismantling of the cytoskeleton
Organelle function	• Organelles are completely degraded by proteases	• Organelle function ceases, but organelles are not completely degraded
Final fate of cell corpses	• Dead cells are regularly sloughed off into the environment as squames after proteolysis of cell-cell contacts	• Apoptotic cells are phagocytosed by macrophages

Table 1 is based on Lippens et al. 2005, Candi et al. 2005 & Allombert-Blaise et al. 2003

Reptilian epidermis cornifies in a parakeratotic manner. That means that the nuclei of keratinocytes are still present in the *stratum corneum*. In mammals however, parakeratosis is clearly a pathological feature. Thus, cornification in reptiles may be described as a more primitive modality of cornification, where apoptosis or apoptosis-like processes appeared in conjunction with the formation of the *stratum corneum* [Alibardi 2003].

Both ways of keratinization are also present in birds, which are evolutionary related to reptiles. Avian epidermis comprises scaled and non-scaled areas. A soft interfollicular epidermis (*apterilae*) and a feathered epidermis (*pterylae*) can be distinguished, while scales are only present in the skin of the hind limbs [Alibardi & Toni 2006, Alibardi 2005]. Apteric epidermis is similar to mammalian epidermis but has no evident granular layer (which is the same in mammalian hard-cornified tissue). However, cross-reactivity with antibodies against mammalian cornification markers (α-keratins, Loricrin, Filaggrin, transglutaminases) has been demonstrated as well as the formation of cornified and lipid envelopes [Alibardi & Toni 2006, Alibardi 2005]. This indicates that keratinization and cornification in avian apteric epidermis roughly resembles the respective mammalian process. According to the literature, the lack of the *stratum granulosum* with its keratohyalin granules results in a poorly developed interkeratin matrix [Alibardi & Toni 2006] that might be responsible for the soft character of avian apteric epidermis.

Table 2: Comparison of cornification processes in amphibians, reptiles, birds and mammals.

	Amphibians	Reptiles	Birds	Mammals
Str. corneum	Replacement layer	α-kertinization: thin β-kertinization: thick (occur at the same sites)	α-kertinization: thin β-kertinization: thick (occur at different sites)	thick layer; only α-kertinization
Str. granulosum	No	yes	no	yes
Shedding	Moulting	moulting or wearing	shedding?	shedding
Keratinization-Type	A	α+β	α+β	α
CE	--	++	++	++
HPRs	No	Loricrin Filaggrin	Loricrin Filaggrin	Loricrin, Involucrin, Filaggrin
Parakeratosis	Yes	Yes	?	Pathologically

Table 2 is based on Alibardi & Toni 2006, Alibardi 2005 and Alibardi 2002. Str. = Stratum, ? indicates lack of information

β-keratinization occurs in the scaled regions of the epidermis and during production of the feathers. β-keratins are similar to those of reptiles. Despite these similarities, the exact mechanisms of cornification in birds remains to be elucidated [Alibardi & Toni 2006].

As only anamniotes amphibian epidermis also constitutes a *stratum corneum* [Alibardi 2009]. Depending on the post-metamorphic adaptation, two different types of amphibian epidermis exist. In species which remain permanently aquatic, no cornified layer is formed and the epidermis is purely mucogenic. In terrestrially adapted forms, one ore two layers of cornified epidermis are formed on top of the stratified epidermis [Alibardi 2009]. Cells from the basal layer differentiate and eventually form a replacement layer that replaces the corneous layer during moult [Alibardi 2003b]. The process of moulting is under strict hormonal control (e. g. aldosterone). The dead cornified layer is shed and the granular layer underneath completes cornification [Smith 1975]. Interestingly, electron-dense granules are present in the upper spinous layer during epidermal differentiation. The smaller granules with a mucus-like content are discharged into the extracellular space, whereas the larger granules with an unknown content stay inside the keratinocytes [Farquhar & Palade 1964]. These granules are believed to contain mucus and crosslinking-proteins (that are different from the mammalian proteins) and form intracellularly a dense mucus interkeratin matrix [Alibardi 2003b, Navas et al. 1987, Bueno et al. 1981]. However, there is a lack of information about interfilamentous matrix proteins in the epidermis of non-mammalian vertebrates [Alibardi 2003b, Matoltsy 1987]. At least a cornified envelope does not seem to be formed in amphibians [Alibardi 2002].

In summary, cornification as it occurs in mammals is probably the most advanced form in terms of α-keratinization, but many different - perhaps more primitive forms - can also be found throughout the animal kingdom. The main differences that are already known are listed in **table 2**.

Teleost epidermis

In vertebrate epidermis two different developmental pathways have been demonstrated, which are tightly linked to environmental adaptation. One pathway of this duality leads to keratinization, occurring predominantly in land-living animals, whereas the other one ends in mucogenesis and is common in amphibious or aquatic vertebrates [Henrikson & Matoltsy 1967]. Due to its aquatic environment, the epidermis has to protect fish from osmotic pressure stress, physical damage and infectious organisms including parasites [Hawkes 1974]. The epidermis of adult individuals is pluristratified and organized in three distinct strata: the basal layer on top of the basement membrane, the intermediate layer and the outer superficial layer [LeGuellec et al. 2004] (**figure 2**). In contrast to mammalian epidermis, living epidermal cells are in direct contact with the environment [Hawkes 1974]. This means fish skin consists exclusively of living cells [LeGuellec et al. 2004]. Processes like keratinization and cornification that are characteristic for land-living vertebrates are absent in fish [Burgess 1956] or occur only at specific sites, as for example adhesive organs, lips or breeding tubercles. Generally, the surface of the skin is covered by mucus but not dead cornified cells [Chang & Hwang 2011, LeGuellec et al 2004].

Ontogenetically, the cells of the basal and intermediate layer derive from the ectodermal sheet after gastrulation, whereas the origin of the superficial layer not completely understood. It is considered to develop from the embryonic enveloping layer (EVL), which derives from the blastoderm during blastula period [Chang & Hwang 2011, LeGuellec et al. 2004].

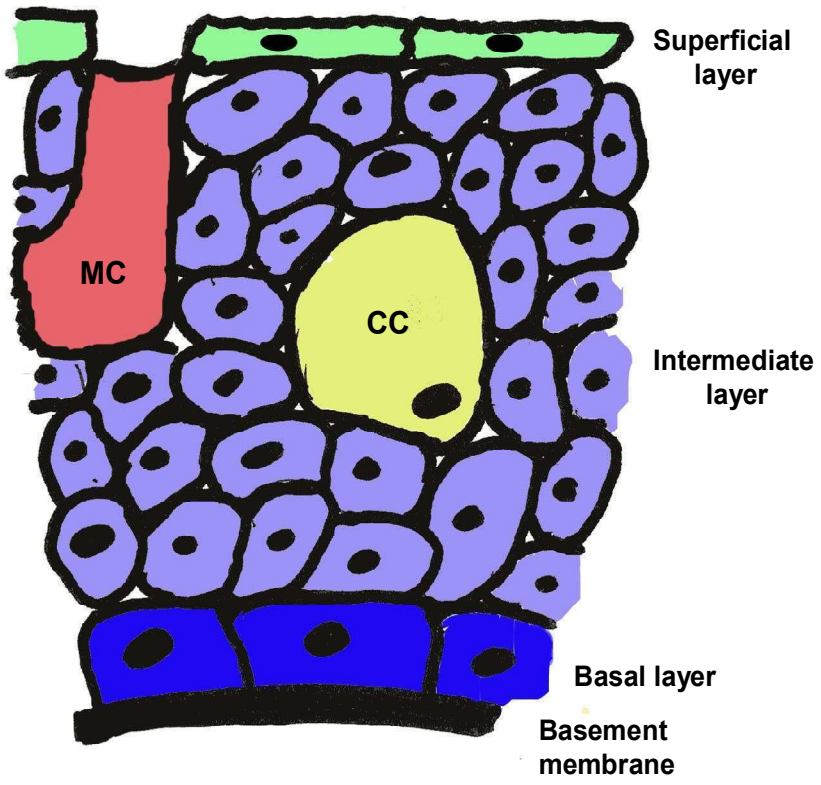


Figure 2: Anatomy of teleost epidermis. Three different compartments can be distinguished in the epidermis of teleosts: the basal layer, the large intermediate layer with mainly undifferentiated cells, and the flat superficial layer. The intermediate layer also comprises certain differentiated cell types as mucous cells (MC) or club cells (CC). Ionocytes are not shown.

Even if fish epidermis is also a pluristratified epithelium, the high degree of organization as seen in mammalian epidermis can not be detected in teleost epidermis. Whereas in mammals basal, spinous, granular and horny or cornified cell layers can be distinguished, which resemble the degree of differentiation of the individual keratinocytes of the certain layers, fish epidermis consists mainly of undifferentiated cells in the

intermediate layer [Chang & Hwang 2011, LeGuellec et al. 2004]. However, the epidermis of fish comprises many differentiated cell types that do not exist in mammalian skin at all, for example mucous cells, club cells or ionocytes. On the other hand, those cells are structurally similar to some mesoderm- or endoderm-derived cells in mammals. For example mucous cells share similarities with mammalian goblet cells of the intestine epithelium and ionocytes have features needed for ion- and osmolarity-regulation, which are similar to mammalian epithelial kidney cells [Chang & Hwang 2011].

In mammalian skin, mitosis only occurs in basal cells. In teleost skin, autoradiographic studies with tritiated thymidine showed incorporation into cells throughout all epidermal layers indicating that in fish epidermis, mitosis is not restricted to the basal layer but occurs throughout the whole intermediate layer and the superficial layer as well [Henrikson & Matoltsy 1967].
In land-living tetrapods, epidermal cells adopt specific structural changes as they undergo terminal differentiation. They synthesize differentiation products and become transformed into protective corneocytes when they reach the distal *stratum corneum*. In regular fish skin, a comparable differentiation process does not seem to exist. It is assumed that density and bundling of keratin filaments do not differ in basal and intermediate layers. In mammalian skin, formation of tonofibrils is a hallmark of differentiation, whereas in teleost skin, filaments remain discrete rather than aggregate, as shown in electron microscopy studies. Most of these cytoskeletal rearrangements in mammals occur in the granular layer that is characterized by the presence of hyaline granula (keratohyalin). This cannot be found in fish skin either [Henrikson & Matoltsy 1967].

The outer surface layer, the superficial layer, is a single cell layer of flat viable cells. These cells develop microridges at the outer surface and are rich in keratins filaments. However, in contrast to mammals these cells do not get keratinized or cornified [Chang & Hwang 2011, LeGuellec et al. 2004]. The cells of the superficial layer are not shed and renewed regularly, but replaced individually after cell death [Chang & Hwang 2011].
Microridges are small protrusions (0.5 – 1 µm height) of the cell membrane, which are similar to microvilli of the intestine epithelium. They are formed by the actin cytoskeleton with the actin filaments being arranged perpendicular to the cell surface and an attachment plate at the basis of the microridge. These cellular protrusions are thought to protect fish against traumata and retain secreted mucus (which protects against various microbia) at the skin surface. Additionally, they might be needed during initial wound closure since the microridges can move by the contraction of their basal microfilaments [Bereiter-Hahn et al. 1979, Hawkes 1974].
In contrast to mammalian epidermis, tight junctions in fish epidermis are found only between the cells of the superficial layer that means in the outermost layer of the epidermis [Henrikson & Matoltsy 1967]. The origin of the superficial cells is not clear. However, they share many similarities with EVL (enveloping layer) cells which cover the embryo from blastula period on [Chang & Hwang 2011], whereas the intermediate and basal cells originate from the embryonic ectoderm [Chang & Hwang 2011, LeGuellec et al. 2004].

The large intermediate layer is composed of different cell types. The major components are relatively small basophilic cells [Henrikson & Matoltsy 1968] which resemble undifferentiated epidermal cells or keratinocytes with a low level of differentiation [Chang & Hwang 2011, LeGuellec et al. 2004]. They operate as a reservoir to replace dead cells of the epidermis.
The undifferentiated cells can divide rapidly and replace dead cells [Chang & Hwang 2011, LeGuellec et al. 2004]. The number of layers of the intermediate layer is highly variable and differs between different body regions [LeGuellec et al. 2004]. The cells are tightly connected to each other by numerous desmosomes [Henrikson & Matoltsy 1967].
The regulation of keratinocytes differentiation in fish skin remains still unclear [Chang & Hwang 2011]. Tonofibrils are described for fish keratinocytes but are only scarcely present compared to mammals and do not form a typical tight network [Hawkes 1974]. Additionally all histidine-rich proteins that are important for cornification (Involucrin, Loricrin and Filaggrin) are not existing in fish genomes and no incorporation of tritiated histidine could be detected by autoradiogaphy in contrast to mammalian or reptilian epidermis. [Alibardi 2003, www.ensembl.org]. Furthermore, no specific keratin expression has been reported in teleost epidermis so far. That means a "keratin-code" which is typical for mammalian epidermis is not known for fish epidermis so far. However, in general fish keratins are very different from mammalian keratins [Schaffeld et al. 2007].
Additionally to keratinocytes, different types of unicellular glands occur in the intermediate layer like mucous cells or club cells, sensory cells and ionocytes [Chang & Hwang 2011]. Each cell type has specific functions.
Mucous cells produce mucus substances that cover the outer surface of the fish and protect it from infections [Chang & Hwang 2011]. Mucous gland cells are basally derived cells that move upwards through the epidermis and thereby displace the boundaries of the adjacent cells [Hawkes 1974]. During their migration, the cells increase remarkably in size, until finally engorged cells reach the surface where they discharge their content into the extracellular space [Henrikson & Matoltsy 1968 b]. Club cells release alarm signal factors into the surrounding and exhibit a fright reaction. However, they are considered to have slightly different functions in different species [Chang & Hwang 2011].
Ionocytes carry specific ion transporters located at the cell membrane and are necessary for homeostasis and regulation of osmolarity of the body fluids [Chang & Hwang 2011]. This type of glands seems to develop from the same precursor population as the keratinocytes. After gastrulation, precursor cells start expressing the transcription factor ΔNp63. In few cells, which will develop into ionocytes, the transcription factor foxi3a and deltaC are turned on whereas ΔNp63 is downregulated. Notch signalling in the surrounding cells together with sustained ΔNp63 expression drives these undifferentiated cells into the keratinocytes lineage [Chang & Hwang 2011].
The basal cell layer is a single cell layer that is attached to the underlying basement membrane via hemidesmosomes [LeGuellec el al. 2004, Mittal & Whitear 1979]. The basement membrane separates the epidermis from the underlying dermis. In fish epidermis, the main function of the basal layer is the tight connection of the epidermis to the dermis [Chang & Hwang 2011, LeGuellec et al. 2004]. However, the basal cells themselves are connected to each other only by a relatively small number of

desmosomes [Mittal & Whitear 1979]. During skin development, the basal layer is involved in the production of the initial collagenous stroma of the dermis [LeGuellec et al. 2004]. In contrast to amniote epidermis, the basal layer in fish epidermis contains non-differentiated and differentiated cells [Chang & Hwang 2011].

Taken together, despite some similarities, fish epidermis and the epidermis of land-living vertebrates are quite different in architecture and composition.

Breeding tubercles

Breeding tubercles (also described as pearl organs) are spine-like epidermal structures found in at least 15 families within four orders of teleosts (especially cypriniformes) [Chen & Arratia 1996] and are thought to facilitate contact between individuals during spawning [Wiley & Colette 1970]. Accordingly, they frequently exist in body regions that come in contact with other fish, for instance head, fins or flank. Additionally, some species use them for nest or territory defence, or stimulation of females during breeding. While breeding tubercles are purely epidermal structures, analogous spikes which include also the dermis are also described in a variety of species. In contrast to breeding tubercles, these are classified as contact organs. In most cases, breeding tubercles as well as contact organs have a sexually dimorphic pattern in males and females. Often breeding tubercles are only or predominantly found in males [Wiley & Colette 1970].

Already fifty years ago, it became obvious that the cells of breeding tubercles consist of a substantial amount of keratins compared to lower levels of keratin in the remaining fish epidermis [Wiley & Colette 1970]. Light and electron microscopy studies revealed a different composition of the epidermal cells in these tubercles including a process of keratinization in the outer cells and subsequent cell death at the outer surface [Mittal & Whitear 1979, Wiley & Colette 1970]. This is in clear contrast to the general agreement that keratinization and a terminal differentiation process are absent in fish skin.

In contrast to normal fish skin, mitosis is expected to occur in a *stratum germinativum* (in mammalian epidermis: basal layer + lower spinous layer) directly above the basement membrane [Mittal & Whitear 1979, Wiley & Colette 1970]. The cells of the layers above become progressively hypertrophic and polygonal with large nuclei toward the outer surface. The outermost layer of the breeding tubercle appears keratinized with high amounts of keratin as shown by histology staining and electron microscopy [Mittal & Whitear 1979]. The transition between the hypertrophic cells and the outer keratinized cells is so abrupt that no transition zone is usually visible. In the keratinized cells, the nuclei disappear completely or persist as pyknotic remnants in the flattened, irregular cells of the keratinized layer [Mittal & Whitear 1979]. The general appearance of a breeding tubercle is summarized in **figure 3**.

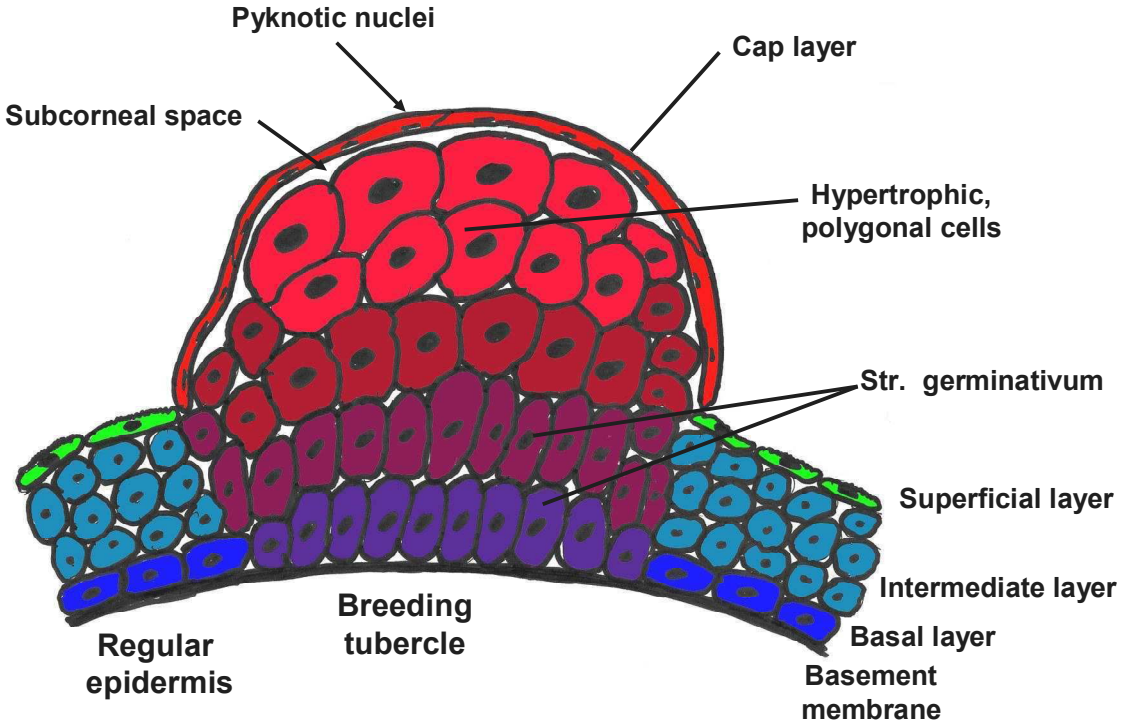


Figure 3: General features of breeding tubercles. The general organization and the major differences of breeding tubercles to regular epidermis are summarized here. Please refer to text for details.

In general, breeding tubercles develop prior the spawning season and reach their maximum size right before the season starts. After the spawning period is over, they break off, slough off or regress gradually. The development of the breeding tubercles seems to be affected mainly by the gonads and the pituitary gland [Wiley & Colette 1970], as gonadectomy prevents the formation of tubercles in the goldfish, and intraperitoneal injection of pituitary extract causes tubercle growth in minnows [Wiley & Colette 1970, Ramaswami & Hasler 1955].

In amniotes and amphibia, the process of cornification is characterized not only by the presence of bundled keratin, but also involves death of cornified cells. A sloughing layer of dead but highly keratinized cells was found at the surface of certain elevations of the epidermis of the Indian catfish Bagarius bagarius [Mittal & Munshi, 1970]. These elevations showed all the features described for breeding tubercles, even if they not present with the classical breeding tubercle shape. In 1979, Mittal and Whitear used electron microscopy to further characterize the nature of keratinization and cell death in epidermal elevations compared to the non-keratinized epidermis of the furrows between the elevations [Mittal & Whitear 1979]. This study showed that basal cells below the elevations were bigger and more cubical than in the furrows. A striking feature was an abrupt transition in the appearance of keratinocytes between the fifth and sixth tier from the surface. From the fifth tier (from top) on, tonofibrils were condensed into bundles changing shape and staining behaviour of the cells. This transition was explained by the authors by an increase in calcium content, shown by alicarin-S staining. Starting in the second tier, the cells flatten and the tonofibril bundles orientated parallel to

the cell surface at least in the distal cytoplasm. The cells of the second tier were the most superficial living cells of the elevations. They were connected to one another by tight junctions, which normally occur only in the superficial layer in fish epidermis and to the upper tier of dead and keratinized cells by large desmosomes. A typical subcorneal space, as found in amphibians, was detected between the first and second tier in this study and large lipid inclusions were reported for the second tier [Mittal & Whitear 1979]. The outermost cell layer showed the strongest reaction for calcium, indicating its keratinized nature. Additionally, those cells lacked all cell organelles and had an electron-dense layer of unknown material at the inner side of the plasma membrane, reminding of the cornified envelope in mammalian epidermis [Mittal & Whitear 1979].
Openings of mucous glands were never found in the keratinized regions of the elevations [Mittal & Whitear 1979], indicating that the furrows resembled a mucogenic type of epidermis, whereas the elevations might be keratogenic. Furthermore, the keratinocytes in those regions were capable of synthesis of mucous granules [Das & Nag 2006]. In fish and amphibian epidermis, large mucous granules are expected to be involved in the clumping of keratin filaments [Fox 1986, Whitear 1986, Budtz & Larsen 1975, Parakkal & Matoltsy 1964].

Other potentially keratinized structures in fish are the horny teeth of the lamprey [Alibardi & Segalla 2011], the horny upper and lower jay sheet of Puntius sophore [Tripathi & Mittal 2011], or the organ of attachment of the catfish Garra gotyla [Das & Nag 2006], which all show many similarities on cellular or histological level. Comparison of morphology and histology of breeding tubercles among different species revealed a high number of significant differences, indicating that tubercles evolved independently in different groups [Wiley & Colette 1970]. However, the process of putative keratinization in the different described structures of fish skin seem to be basically the same or at least similar [Wiley & Colette 1970].

The p53-familiy member p63 and its role in mammalian epidermal development and maintenance

So far, three members of the p53-transcription factor family have been identified: p53, p63 and p73, all with high sequence and structural similarity, but apparently quite different functions [Koster & Roop 2004]. All three proteins share an N-terminal transactivation domain (TAD), a central DNA binding domain (DBD) and a C-terminal oligomerization domain (OD) [Vanbokhoven et al. 2011, Candi et al. 2007, Strano et al. 2001]. Sequence similarity is particularly high between DBD of p63 and p73 but also p53 shares at least 65% similarity with other family members [Melino et al. 2002, Yang et al. 2002, De Laurenzi & Melino 2000].
The most commonly known member of the p53-family is the transcription factor p53 itself which is one of the best studied tumor suppressors and is involved in cell cycle arrest, DNA repair and leading cells into apoptosis [Riley et al. 2008]. As p53 -/- mutants (e. g. mouse mutants or zebrafish mutants)

[Molchadsky et al. 2010, Parant et al. 2010, Tyler et al. 1994] show no visible developmental defects but develop tumors later during adulthood, p53 is believed to be dispensable for development but very important to avoid tumor formation.

Different cellular stress scenarios, like DNA damage, spindle damage or hypoxia activate p53, which levels are tightly controlled via MDM2, an ubiquitin ligase, driving p53 into proteasomal degradation [Riley et al. 2008]. An activated p53 tetramer binds to p53 responsive elements (RE) in the promoter region of certain p53 target genes and thereby alternates gene expression of the cell inducing DNA repair, cell cycle arrest, cellular senescence or apoptosis [Riley et al. 2008].

Furthermore, p53 seems to be involved in asymmetric cell division of stem cells, giving rise to a dividing daughter cell and a postmitotic cell arrested in G1/S of the cell cycle [Sherley 2002]. As these results were shown mainly by *in vitro* experiments, the significance of this hypothesis still has to be demonstrated *in vivo*. However, this finding is consistent with the well-established idea that increased symmetric cell divisions increase the risk of carcinogenesis, as it was shown in p53 null animals [Sherley 2002]. Additionally, cultured explanted cells from p53 null mice do no longer show signs of senescence. The absence of senescence by cultures of p53 null tissue cells is consistent with abrogation of the asymmetric cell kinetics barrier to adult stem cell expansion by removal of p53-dependent regulation [Sherley 2002, Rambhatla et al. 2001, Merok & Sherley 2001].

On the RNA level, different p53 isoforms are transcribed by three different promoters in the p53 gene. Additionally, alternative splicing at the 3'-end, leads to sets of α-, β- & γ- isoforms [Bourdon et al. 2005, Rohaly et al. 2005]. However, the full length protein is the most abundant isoform, and the alternative mRNAs seem to be produced especially under pathologic conditions (e. g. in tumors) [Rohaly et al. 2005]. The promoter P1 transcribes the regular p53 isoforms, whereas the alternative P1' promoter leads to truncated Δ40p53 isoforms and the promoter P2 (upstream the fifth exon) to Δ133p53 isoforms. The functions and importance of the different isoforms remain largely unknown [Rohaly et al. 2005].

In contrast, the different isoforms of p63 and p73 are investigated in much more detail [Strano et al. 2001]. An alternative promoter upstream the third exon (in mammals) gives rise to two different types of isoforms: TA-isoforms with an N-terminal transactivation domain and ΔN-isoforms without this domain [Strano et al. 2001, Koster & Roop 2004]. Additionally, alternative splicing at the 3`-end contributes to a more complex set of isoforms with the same 5`-end but different 3`-ends [Strano et al. 2001]. The longest set of isoforms with the same 5'-end, named α-isoforms, carry an additional sterile alpha motif (SAM) domain at the 3'-end. This domain often contains protein interaction motifs frequently found in proteins that are involved in development and differentiation [Koster & Roop 2004], suggesting a role for p53-familiy members in such processes. Smaller sets of isoforms, the β- and γ-isoforms, lack the SAM domain [Vanbokhoven et al. 2011, Candi et al. 2008, Koster & Roop 2004]. p53, on the other hand, does not have a SAM domain at all [De Laurenzo & Melino 2000].

The diversity of p63 isoforms gets even more complicated by different transcriptional and translational starts in the 5'-region. Bamberger and Schmale identified four different ATGs (AT1-4) in the TA specific region leading to four different sets of isoforms [Bamberger & Schmale 2001].

The domains of the most important isoforms of p53 and p63 are shown in **figure 4**.

In contrast to p53, p73 and especially p63 do not have a remarkable function as tumor suppressor, but have specific functions during development [Ramadan et al 2005, Moll & Slade 2004, Di Laurenzi & Melino 2000]. As much more information is available for p63, the following description refers primarily to p63 which was shown to have crucial functions during formation of the epidermis.

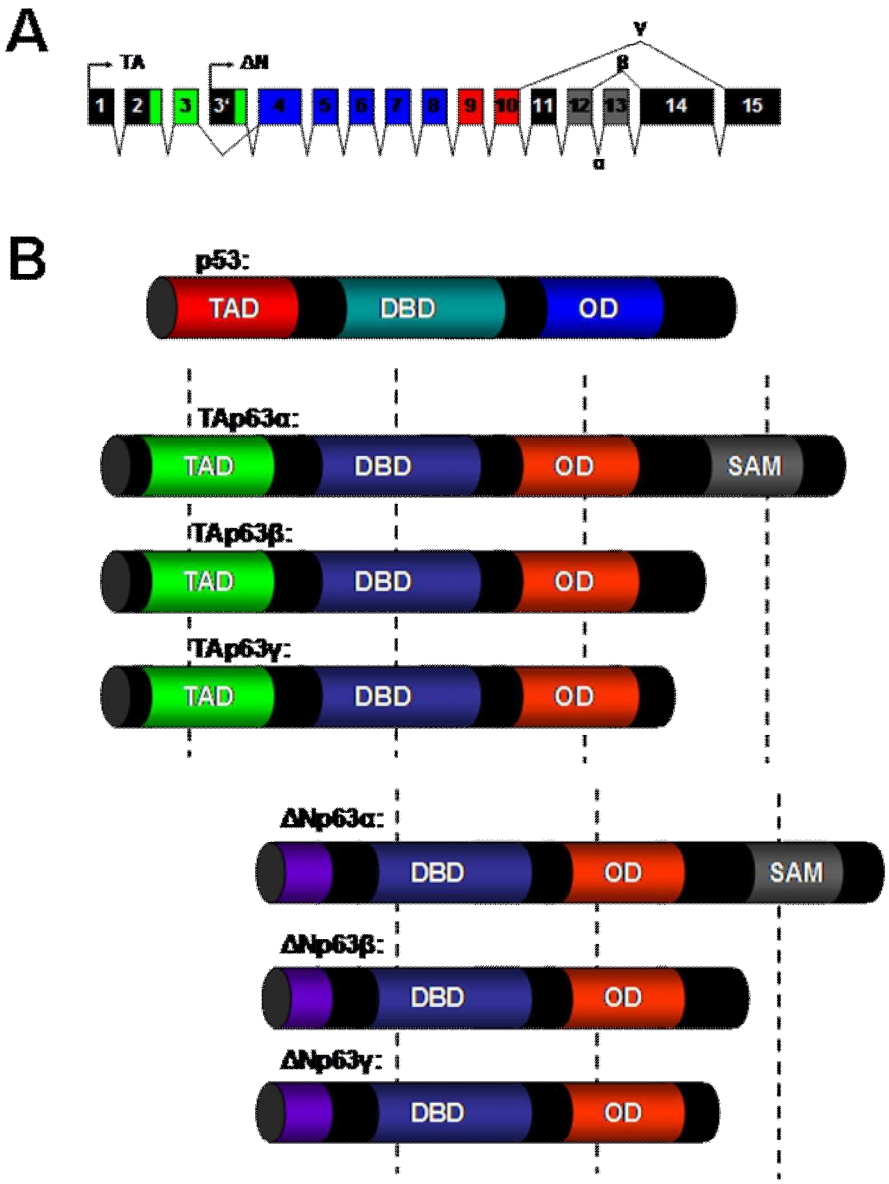


Figure 4: Organization of p53 and p63. (A) Genomic organization of the mammalian p63 gene. p63 consists of a 3'-transactivation domain (TA, green), a DNA binding domain (DBD, blue), and an 5'-oligomerization domain (OD, red). According to a second promoter, truncated ΔN isoforms are generated lacking the N-terminal transactivation domain. Alternative splicing at the 3'-end gives rise to additional sets of isoforms with the same 5'-end, named α-, β-, and γ-isoforms. Only the long α-isoforms have an additional sterile alpha motif (SAM, grey) domain. Splicing variants generated by alternative splicing at the 5'-terminus are not shown. (B) Structure of p53, TAp63, and ΔNp63 proteins. p53 and p63 share high similarity in the DBD and the OD. TAp63 and p53 both have a transactivation domain to activate specific target genes. According to alternative splicing, different truncated versions exist especially for p63, whereas those versions of p53 occur predominantly only under pathological conditions.

According to initial *in vitro* transactivation studies, the ΔN-isoforms were supposed to be only dominant negative repressors of the respective TA-isoforms [Koster & Roop 2004, Koster et al. 2004, Yang et al 1998] because of the missing 5'-transactivation domain. However, newer results claim also transactivation functions for the different ΔN isoforms possibly due to other transactivation domains [Vanbokhoven et al. 2011, Koster & Roop 2004, Wu et al. 2003, King et al. 2003, Ghioni et al. 2002, Dohn et al. 2001]. Furthermore, it is still not clear whether the dominant-negative function of the ΔN-isoforms results from direct competition for p53-family binding sites in the promoter regions of target genes or by heterotypic dimerization [Koster & Roop 2004, Yang et al. 2002, Gaiddon et al. 2001].

The epidermis in mice (or mammals) develops from the embryonic ectoderm initially as a single-layered epithelium. Until day E9.5, this simple epithelium expresses the respective markers of simple epithelia, keratins 8 and 18. On day E9.5, the epithelial cells commit towards a stratified epithelium, which is demonstrated by a switch from K8/K18 to the basal marker keratins K5/K14. At day E10.5, the epidermis becomes bilayered. A layer of flat cells, the periderm, develops on top of the basal layer [Koster & Roop 2004b]. In contrast to the EVL in fish, the mammalian periderm originates directly from the basal layer of the epidermis. The periderm is needed for protection while the embryo is still in the amnion cavity. However, as in fish EVL, the first tight junctions of mammalian epidermis develop between cells of the new periderm at around day E11. These initial tight junctions are more similar to tight junctions of simple epithelia than to *maculae occludentes* in the granular layer of the mature stratified epidermis [Morita et al. 2002, M'Boneko & Merker 1988, Nakamura & Yasuda 1979]. At day E13.5, the epidermis eventually stratifies. In the following, an intermediate cell layer forms between the basal layer and the periderm. Initially this second layer proliferates as well, probably due to the rapidly growing embryo. But soon later at day E15.5, the cells in this layer become postmitotic and start to differentiate, what is reflected in the switch towards K1/K10, the keratins of suprabasal cells [Koster & Roop 2004b, Weiss & Zelickson 1975]. Calcium signalling is needed for the commitment as well as for the later differentiation of keratinocytes [Koster & Roop 2004b]. Cultured primary human keratinocytes proliferate and remain features of basal cells only when kept at low calcium concentration. When the concentration is increased, cells withdraw from cell cycle and start terminal differentiation. During epidermal development, a calcium gradient is established around the time, the spinous layer occurs [Koster & Roop 2004b, Elias et al. 1998]. In accordance with these results, Okuymama et al. demonstrated that E15.5 epidermal keratinocytes have an intrinsically higher commitment to terminal differentiation than newborn epidermal keratinocytes [Koster & Roop 2004b, Okuyama et al. 2004].
Until day E19.5, the epidermis gets multilayered, always with viable periderm cells on top. At a final step in epidermal development, the periderm cells are shed at E19.5 and are replaced by the *stratum corneum* with fully differentiated and dead keratinocytes [Akiyama et al. 1999, Byrne et al. 1994, Holbrook & Odland 1975]. Mammalian epidermal development is summarized in **figure 5**.

The expression pattern of the different p63 isoforms especially during epidermal development is still under intensive discussion. In adult mice, p63 is expressed at high levels in the basal cells of the epidermis and other stratified epithelia. In the suprabasal layers p63 is strongly reduced or completely absent [Pellegrini et al. 2001, Yang & McKeon 2000, Mills et al. 1999]. However, the generation of isoforms-specific antibodies identified only the ΔN isoforms in the basal layer, whereas the TA isoforms were found in the suprabasal layers, indicating a switch during keratinocytes differentiation from the ΔN to the TA isoforms [Nylander et al. 2002]. According to its expression pattern and the phenotype of p63 knock-out mice (described below), p63 was expected to have an essential role during development and/or differentiation of the epidermis [Koster & Roop 2004]. Both, TAp63 and ΔNp63 isoforms were found to be expressed in mouse embryos and in adult mouse epidermis as shown by RT-PCR using isoforms-specific primer. In detail, this expression analysis of whole embryos (E7.5-E9.5) and embryonic epidermis (E15.5-E18.5) demonstrated the presence of TAp63 as early as E7.5, prior to commitment to stratification. In this study, ΔNp63 transcripts were not detected prior to day E9.5, with TAp63 still being the predominant isoform until day E18.5 [King & Weinberg 2007, Koster at al. 2004]. Additionally in support of the early onset of TAp63, it was shown that ectopic expression of TAp63 but not ΔNp63 in single layered lung epithelium was necessary and sufficient for initiation of a stratification program demonstrated by expression of K5/K14 [Koster & Roop 2004, Koster et al. 2004].

However, a different study could not find any p63 transcripts at E7, and only ΔNp63 at E8 and E9 [King & Weinberg 2007, Laurikkala et al. 2006]. Similarly in zebrafish, ΔNp63, but not TAp63, expression was observed and reported to be required for morphogenesis of the stratified epidermis [King & Weinberg 2007, Lee & Kimmelman 2002]. qRT-PCR data of skin samples from mouse embryos during different stages of skin development, while the epidermis is already bilayered (day E13), are available. These data revealed that 99% of the existing p63 transcripts were ΔN isoforms, whereas only 1% was identified as TA isoform [King & Weinberg 2007, Candi et al. 2007].

To make it even more complicated, *in situ* hybridization studies with isoforms-specific antisense probes could show that only the ΔN isoforms but not the TA isoforms were expressed in epithelia of mice from day from E11 to E14 [Laurikkala et al. 2006]. Additionally, isoforms-specific antibodies detected only the ΔN isoforms but not the TA isoforms of p63 during epidermal development (E10.5-E16.5) [Romano et al. 2009].

In summary, it is still not clear if TAp63 is expressed in embryonic skin at all [Vanbokhoven et al. 2011, Livera et al. 2008, Suh et al. 2006]. It became clear, that the ΔN-isoforms are the more abundant isoforms compared to the TA-isoforms at the protein level [Vanbokhoven et al. 2011, Koster et al. 2007]. ΔNp63 is highly expressed in basal keratinocytes, but downregulated in suprabasal cells. TAp63 is strongly expressed in oocytes, but expression in the epidermis is still unclear [Vanbokhoven et al. 2011, Livera et al. 2008, Suh et al. 2006].

Mutations in the human p63 gene are responsible for several ectodermal dysplasia syndromes (EDS) that are congenital disorders with abnormalities in ectodermal derived structures like hair, teeth, nails

or even craniofacial structures or digits [Vanbokhoven et al. 2011]. p63-deficient mice develop no stratified epidermis and have aberrant squamous epithelia (e. g. cervix or urothelium) [Vanbokhoven et al. 2011]. Additionally they lack epithelial appendages like mammary glands, hair follicles or teeth [Vanbokhoven et al. 2011, Koster et al. 2007]. Consequently, p63-deficient mice die shortly after birth, most probably due to dehydration because of the absent barrier function of the skin [Candi et al. 2008]. Besides the epithelial phenotype, also p63 knock-out mice have truncated limbs and cranio-facial defects [Vanbokhoven et al. 2011].

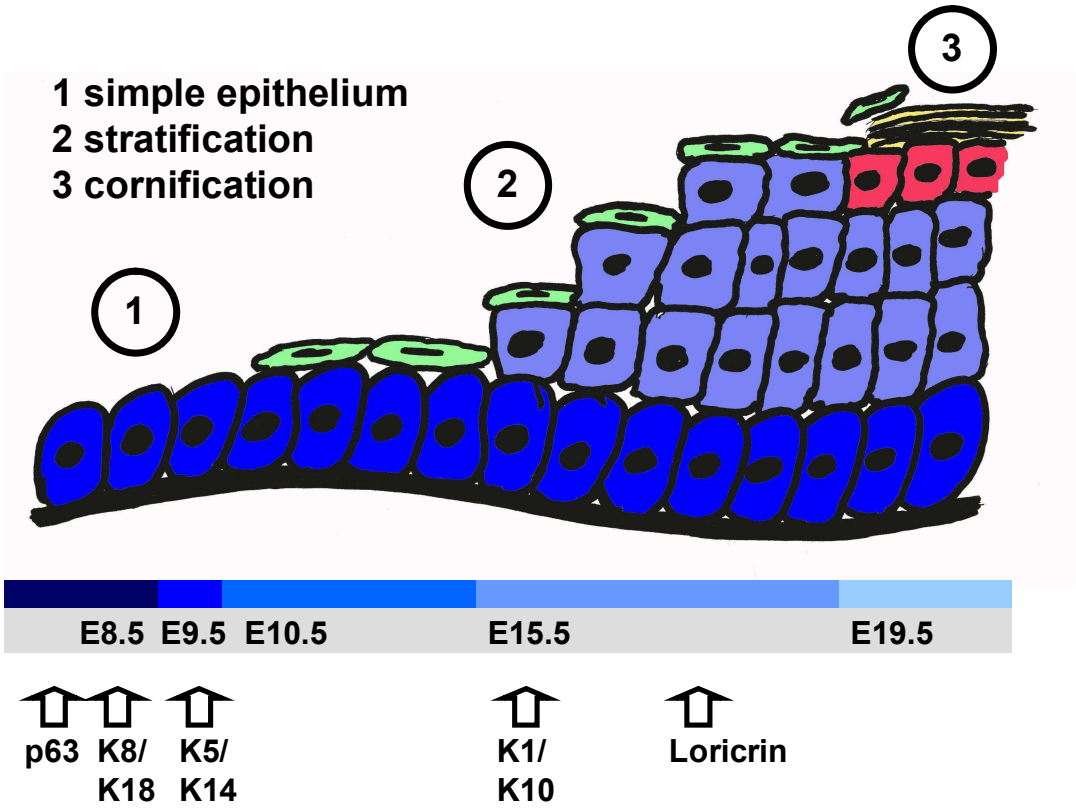


Figure 5: Mammalian development of the epidermis. In mammals, the development of the epidermis starts with a simple epithelium originating from the embryonic ectoderm. After occurrence of the periderm layer, the epidermis stratifies while it still consists exclusively of living cells. At the end of the developmental process the periderm cells are shed and the cornified layer develops. The time frame of the different steps and the most important marker molecules are shown below the figure. Please see text for details. Blue: basal cells, light blue: spinous cells, red: granular cells, yellow: cornified cells, green: periderms cells.

However, the generation of two independent p63-(isoform independent) knock out models led to controversial results concerning the function of p63 in epidermal development. Both strains were generated on different genetic backgrounds with different targeting strategies. On the first sight, both of them had severe defects in epidermal development and lacked epidermal appendages as described above. A subsequent more detailed analysis led to two quite different hypotheses concerning the function of p63 in the epidermis [Vanbokhoven et al. 2011]: Knock-out mice created in the Bradley laboratory were covered by a one-layered simple epithelium [Koster et al. 2007, Mills et al. 1999] that

did not stratify and lacked all specific markers of differentiation. Furthermore, the epithelial cells expressed K8 and K18, typical markers of simple epithelia [Vanbokhoven et al. 2011, Mills et al. 1999]. Consequently, it was concluded that p63 is necessary for epidermal commitment and stratification [Vanbokhoven et al. 2011]. As epithelial cells do not express K5 and K14, the epidermis might remain arrested in a premature state prior to the onset of stratification [Koster & Roop 2004b]. Similarly, it was demonstrated in 2004 that the switch in the differentiation of the Muellerian duct depends on p63. In the absence of p63, the Muellerian duct epithelium develops into a single layered uterine epithelium, while induction of p63 expression results in the differentiation into a cervicovaginal (stratified) epithelium [Koster & Roop 2004b, Kurita et al. 2004]. Additionally, it was demonstrated in this study that p63 expression in the Muellerian duct is induced via signals of the underlying mesenchyme [Kurita et al. 2004], whereas a role of the mesenchyme in epidermal p63 induction is not known so far. According to the similarities in development of the different stratified epithelia, an equivalent role for the mesenchyme in the epidermis is very likely. In support of an early function of p63 in the epidermis, p63 was found to be the first keratinocyte-specific marker in cell culture experiments [Koster & Roop 2004b, Green et al. 2003].
In contrast to the above phenotype, KO mice from the McKeon laboratory presented patches of differentiated keratinocytes that expressed terminal differentiation markers like Involucrin, Loricrin and Filaggrin, placed on an exposed dermis [Vanbokhoven et al. 2011, Koster et al. 2007, Yang & McKeon 2000, Yang et al. 1999]. In accordance with these findings, another study could show that p63 protein is enriched in putative epidermal stem cells [Koster & Roop 2004b Pellegrini et al. 2001]. This led to the interpretation that a stratified epidermis can develop initially in the absence of p63, but cannot be maintained afterwards due to a lack of proliferating cells. According to this hypothesis, p63 would be required for stem cell maintenance but not differentiation [Vanbokhoven et al. 2011].

More recent data indicate that during development and later maintenance of the epidermis, an exactly titrated balance between the different isoforms is absolutely crucial [Mack et al. 2005]. However, additionally to the above described controversial data on p63, especially the TAp63 isoform functions seem to be even more complicated. Different studies claim promoting or inhibitory functions of TAp63 during keratinocyte differentiation and cornification after commitment has occurred.
Cell culture transfection experiments identified TAp63 to directly bind to the Jagged1 promoter and thereby promoting Jagged-Notch signalling [Mack et al. 2005, Sasaki et al. 2002]. Okuyama et al. published data in 2004 where Notch signalling activated Caspase3 in a non-apoptotic manner only during initial cornification in mouse embryonic epidermis. Blocking Notch signalling by the γ-secretase inhibitor DAPT also blocked activation of caspase3. Furthermore, important cornification markers like Loricrin and Filaggrin were significantly reduced in caspase3 knock-out mice [Mack et al 2005, Okuyama et al 2004]. These data claim a differentiation-driving pathway in mammalian epidermis, where TAp63 activates Jagged-Notch signalling. Then Notch enhances terminal differentiation and cornification via a non-apoptotic function of Caspase 3. However, induction of terminal differentiation of keratinocytes is characterized by withdrawal from the cell cycle and alteration of gene expression.

Several Notch pathway genes were identified to be induced after differentiation starts [Koster & Roop 2004b, Nickoloff et al. 2002, Rangarajan et al. 2001]. These data indicate that Notch has several important functions in the epidermis during development and later maintenance.

Furthermore, TAp63 might also have differentiation promoting effects on keratinocytes that are independent of Notch signalling. *In vitro* experiments have shown that p300 directly interacts with TAp63 at its N-terminus and induces expression of $p21^{WAF/Cip1}$, which causes cell cycle arrest [MacPartlin et al., 2005].

The differentiation promoting effects mainly seem to be an effect of the TAp63γ isoform, whereas TAp63α seems to inhibit terminal differentiation [Mack et al. 2005]. Induction of TAp63α resulted in hyperplasia, hyperproliferation and inhibition of terminal differentiation in adult and embryonic mouse skin [Koster et al. 2004]. According to the author's hypothesis, ΔNp63 would be needed to counterbalance this inhibitory effect of TAp63 after commitment of stratification has occurred, in order to allow the cells to respond to maturation and differentiation signals [Koster et al. 2004].

In an attempt to unravel the controversial results of the different isoforms, p63-KO mice (McKeon laboratory) were used to create transgenic KO mice that express either TAp63, ΔNp63 or both under the control of a *krt5* promoter [Vanbokhoven et al. 2011, Koster et al. 2007, Candi et al. 2006]. All transgenic mutants developed patches of differentiated cells but with slight alterations in comparison to the phenotype of KO mice from the McKeon laboratory [Koster et al. 2007, Candi et al. 2006]. In *krt5::ΔNp63* transgenic p63 -/- mice, the epidermal patches expressed only K5 and K14 as differentiation markers, whereas *krt5::ΔNp63; krt5::TAp63* transgenic p63 -/- mice also expressed K1 and Loricrin. However, this expression of suprabasal markers could not be detected in only TA complemented p63-/- mice [Koster et al. 2007, Candi et al. 2006]. Based on these experiments, the authors concluded that ΔNp63 maintains the proliferative and undifferentiated state of the basal epidermis, whereas TAp63 is required for the formation of the suprabasal layer [Koster et al. 2007, Candi et al. 2006]. However as K5 is not active before commitment of keratinocytes to a stratified epidermis, no conclusions could be made concerning a role of the different isoforms during epidermal commitment. These results again mirror the controversial results of previous experiments, but also highlight the necessity of a properly controlled balance between the TA- and ΔN-isoforms for adequate epidermal formation [Koster et al. 2007].
In contrast to these results, Romano et al. reported in 2009 that K5 as well as K14 are direct targets of ΔNp63. In accordance with previous data, only ΔNp63 but not TAp63 could be detected in the epidermis during development as shown by isoforms-specific antibodies. Therefore, the group concluded that ΔNp63 but not TAp63 is responsible for commitment of epithelial cells to stratification, which is highlighted by the switch from K8/K18 to K5/K14 [Romano et al. 2009]. Additionally, p63 KO mice complemented with ΔNp63 under the control of a tetracycline inducible promoter developed patches of completely normal and stratified epidermis [Romano et al. 2009]. In 2012, the same group

published a p63 knock-in mouse line, where the ΔN-specific exon was replaced by GFP, revealing a "specific ΔNp63-KO situation" [Romano et al. 2012]. The homozygous $\Delta Np63^{gfp/gfp}$ mice strikingly phenocopied the total p63 KO mice described by Mills et al. before. Interestingly, closer investigation by immunohistochemistry revealed that in $\Delta Np63^{gfp/gfp}$ at least some cells expressed K5 indicating that these cells committed to stratification. But also cells expressing K5 together with the simple epithelium marker K8 could be detected, leading to the conclusion that those cells got stuck in an intermediate state of identity crisis. Small cell clumps contained cells that even co-expressed K5 and K1/K10, indicating a higher differentiation status. Finally, also late stage differentiation markers like Filaggrin, Involucrin or Loricrin could be detected in E15.5 $\Delta Np63^{gfp/gfp}$ mice. In control mice these markers did not show up at this stage, indicating a premature or accelerated differentiation program in $\Delta Np63^{gfp/gfp}$ mice [Romano et al 2012]. After all, the analysis of $\Delta Np63^{gfp/gfp}$ mice reaffirms the indispensable role of the ΔN isoform of p63 in epithelial biology and confirms that ΔNp63-null keratinocytes are capable of committing to an epidermal cell lineage, but are likely to suffer from diminished renewal capacity and an altered differentiation fate [Romano et al 2012].

An unconditional TAp63 KO mouse line developed by Suh et al. revealed embryos that were viable and showed normal epidermal morphogenesis [Vanbokhoven et al 2011, Suh et al. 2006]. Furthermore, two conditional Cre/loxP activated KO systems of the TAp63 isoform did not lead to any severe epidermal phenotype [Vanbokhoven et al 2011, Guo et al 2009, Su et al 2009].

Combining all these controversial data into an all-embracing model of epidermal development is still one of the leading challenges in this field. Many problems or controversies arose from limitations of the available tools. Perhaps more "primitive" organisms, as the teleost zebrafish that has proven to be a very useful tool in creating mutants or transgenics, might help to collect new data and information, which might also be important for organisms with a more advanced epidermis that is very different from that of fish.

II. Objective

According to previously published data, breeding tubercles seem to be epidermal structures in fish that share similarities with the terminal-differentiation process of keratinocytes in land-living tetrapods. So far, breeding tubercles have not been described in zebrafish. Demonstration of the existence and localisation in zebrafish should be accompanied by analysis using *in vivo* methods (as transgenic lines or mutants), histological and antibody staining methods as well as molecular methods. Furthermore, transmission and scanning electron microscopy should be used to dissect the ultrastructure of breeding tubercles.

The aim of this work was to compare a putative differentiation process of epidermal cells in teleost epidermis to the well characterized cornification process in land-living animals. In particular, the breeding tubercles should be analyzed according to aspects of proliferation, keratinization, formation, differentiation and subsequent cell death as well as sloughing and renewal of the outer layer. Additionally, the epidermal structure of breeding tubercles should not only be compared to cornified epidermis of mammals or amphibians, but also to regular epidermis of teleosts.

As the available data of the function of p63 in embryonic development and later maintenance of the epidermis are highly controversial, a TAp63 specific zebrafish mutant should be exploited to investigate functions of this isoform in a more "primitive" epidermis. Special emphasis was laid on Notch signalling, as TAp63 was shown to activate Notch in mammalian epidermis in a variety of functions. The conservation of the linear pathway TAp63 → Notch/Jagged1 → Caspase3 should be evaluated as well as its importance in breeding tubercles. We propose to dissect the mentioned pathway by using mutants and specific small compound inhibitors with subsequent epistasis analysis by immunohistochemistry methods.

p63 is a member of the p53-family of transcription factors. The founding member p53 itself is believed to have many common targets with p63 but a specific function during development of the epidermis is not established. To identify putative redundant functions of both proteins, a p53 zebrafish mutant was included in the above proposed experiments.

III. Results

Breeding tubercles in zebrafish

According to literature, breeding tubercles are epidermal appendages that are regularly found in teleosts especially at the head, at the fins or even at the outlines of the scales [Chen & Arratia 1996, Mittal & Whitear 1979, Wiley & Colette 1970]. Histological staining of paraffin sections of adult zebrafish revealed tubercle-like structures at two different sites: most prominent, and present in all fish, at the anterior tip of the lower jaw (**figures 6A & 6B**), and smaller, and only in some fish, at the dorsal side of the pectoral fins (**figure 6C**).

At the lower jaw, two super-structures could be distinguished: a plate containing many spikes particularly at the edge and an additional row of single tubercles along the ridge of the jaw (**figures 6A, 6D & 6E**). Pectoral fin tubercles were arranged in one up to three rows above the fin rays in the middle section of the dorsal side of the pecfins (**figure 6C**). Unexpectedly, breeding tubercles were stained by the vital dyes calcein (**figures 6D & 6E**) and methylene blue (shown below). Regular skin in fish was impermeable for both dyes, indicating that the cells of the epidermis at the tubercles were leaky, or cell-cell contacts did not properly seal the organism against the surrounding.

In general, the tubercles of the head and the pectoral fins seemed to be equal types of structures, even if the pecfin tubercles were much smaller. Tubercles presented with many additional cell layers compared to the surrounding epidermis. The cells of the lower layers in the tubercles had the same size as the cells of the surrounding, however especially the cells of the basal layer often had a more cubical shape and were more evenly arranged (**figure 6B**) as in regular zebrafish epidermis. The cells of the outer layers appeared progressively larger and hypertrophic with large round nuclei (**figures 6B & 6F**). As an outermost layer, the tubercles were covered by a thin cap layer, in most cases only one cell layer thick (in rare cases two to three layers). Often the cap layer had lost contact with the tier underneath or was torn apart from the tubercle by the embedding procedure. This might indicate the fragile nature of cell-cell contacts between the cap layer and the next tier (**figures 6A-6C**).

In all histology staining methods, the cap layer stained differently than the lower layers of the tubercle, indicating its different composition compared to the epidermis underneath. The cap layer appeared bright red in Azan stained sections and purple-red in hematoxylin/eosin staining, possibly indicating a keratinized nature of these cells (**figures 6A-6C & 6F**).

DAPI staining clearly demonstrated the existence of nuclei in the cap layer of jaw tubercles (**figure 6G**) as well as pectoral fin tubercles, indicating that the cap definitely consisted of cells, even if cell borders were hardly detectable. The presence of nuclei also ruled out the possibility of a secreted cuticle. In accordance, PAS staining of tubercles showed no glycoprotein-positive reaction of the cap layer, as would be expected for cuticle structures (**figure 6H**).

In contrast to the big and round nuclei of the layers underneath the outermost layer, the nuclei of the cap layer appeared small and flat, often located at the bottom of the cell. This pyknotic appearance of the nuclei might be due to an at least partially apoptotic character of the cells at the outermost edge of the tubercles (**figures 6B, 6F & 6G**).

Adult wildtype zebrafish that lacked all tubercles were not found at all, however, few fish displayed only a very low number of tubercles. Overall, the number of tubercles was very variable. At the lower jaw, the number of tubercles ranged from 20 to up to 60 tubercles per side.

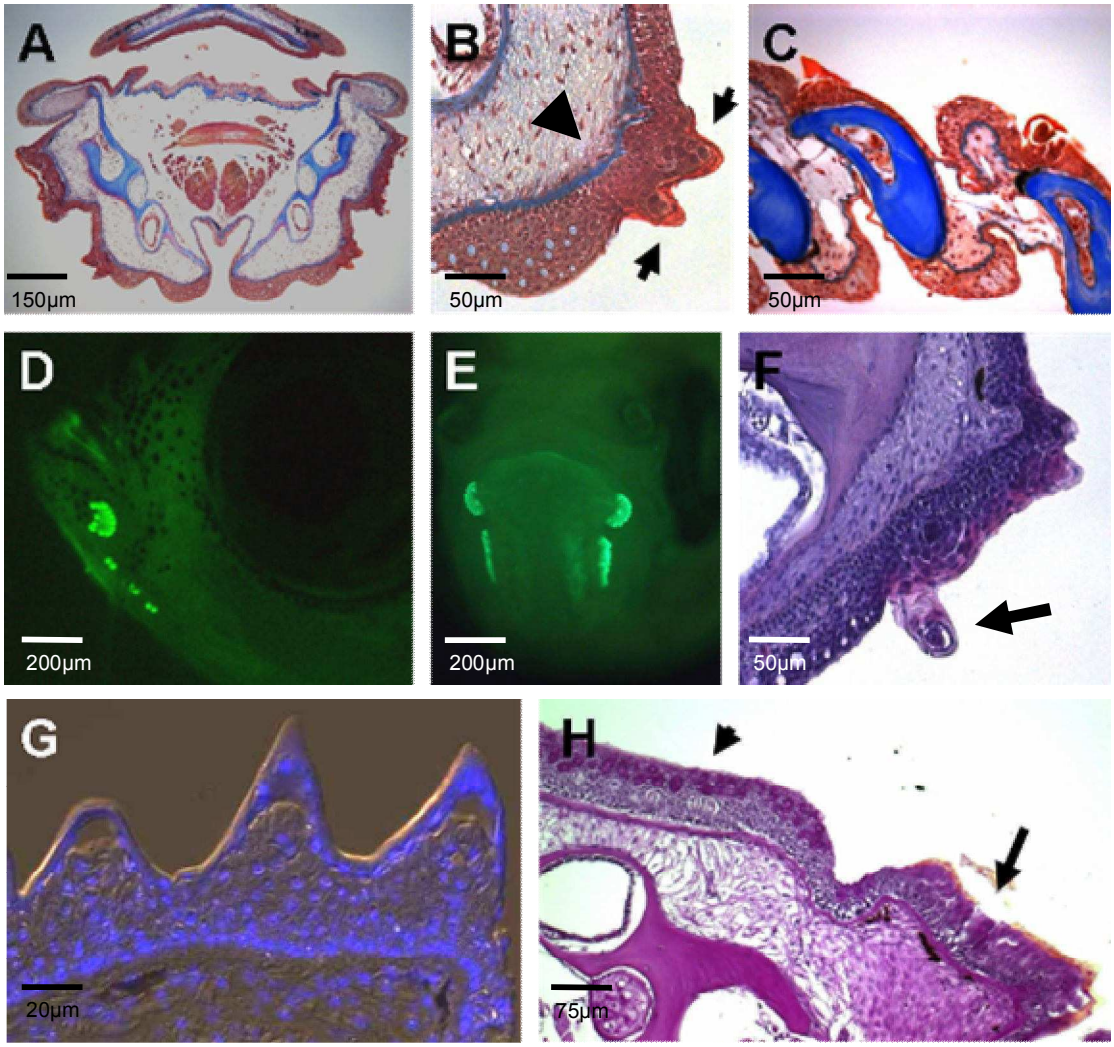


Figure 6: Distribution pattern and general structure of breeding tubercles in zebrafish: (A) Azan trichrome staining of a section of the anterior head. A big plate-like structure with spikes can be seen on both sides. More dorsally, single tubercles can be detected at the lower jaw. The outer cap layer often appeared torn apart from the underlying layers of the tubercle. (B) Magnification of a jaw tubercle. The tubercle has many more layers than the surrounding epidermis with cell shape changes towards the outer top layer, which appears completely flat and reacts differently in histological staining methods (black arrows). The arrowhead shows the more regularly organized basal layer cells in the region of the tubercle. (C) Azan trichrome staining of pectoral fin tubercles. The cap layer above the bony rays has the same staining behaviour as for the head jaw tubercles. (D) Lateral view of calcein stained head tubercles. The plate structure and the dorsal row of tubercles can be distinguished. (E) Frontal view of calcein stained head tubercles. (F) HE stained section of a jaw tubercle at the lower jaw. The purple stained cap layer possibly indicates high amounts of keratin. A wide space between the cap of the spike and the cells underneath is visible (arrow). (G) DAPI staining of the jaw tubercle region. The nuclei of the upper layers of the tubercle are big and roundish, whereas the nuclei of the cap layer appear often flat and condensed indicating a pyknotic state of the cap cells. However it demonstrates that the cap is definitely a cellular tissue. (H) Region of the jaw tubercles stained with the PAS method to detect glycoproteins. Whereas the mucous cells stain bright purple (arrowhead) the cap layer is not stained by this histological staining method, arguing against a secreted cuticle.

Sexually dimorphic pattern of breeding tubercles in zebrafish

Breeding tubercles are often more prominent in male individuals [Wiley & Colette 1970]. In zebrafish, both males and females showed the same distribution of tubercles at the lower jaw. Additionally, it seemed that number and size of tubercles were in the same range in both sexes, as shown by calcein whole-mount staining (**figures 7A & 7B**). On the other hand, pectoral fin tubercles could be detected exclusively in male individuals and never in females (**figures 7C & 7D**), indicating sexual dimorphism. Male and female tubercles always showed the same staining behaviour in all tested staining methods. Even if the distribution of breeding tubercles in zebrafish was different in males and females, the general architecture of the tubercles was identical. Therefore, if not mentioned otherwise, sex was not considered for all following experiments.

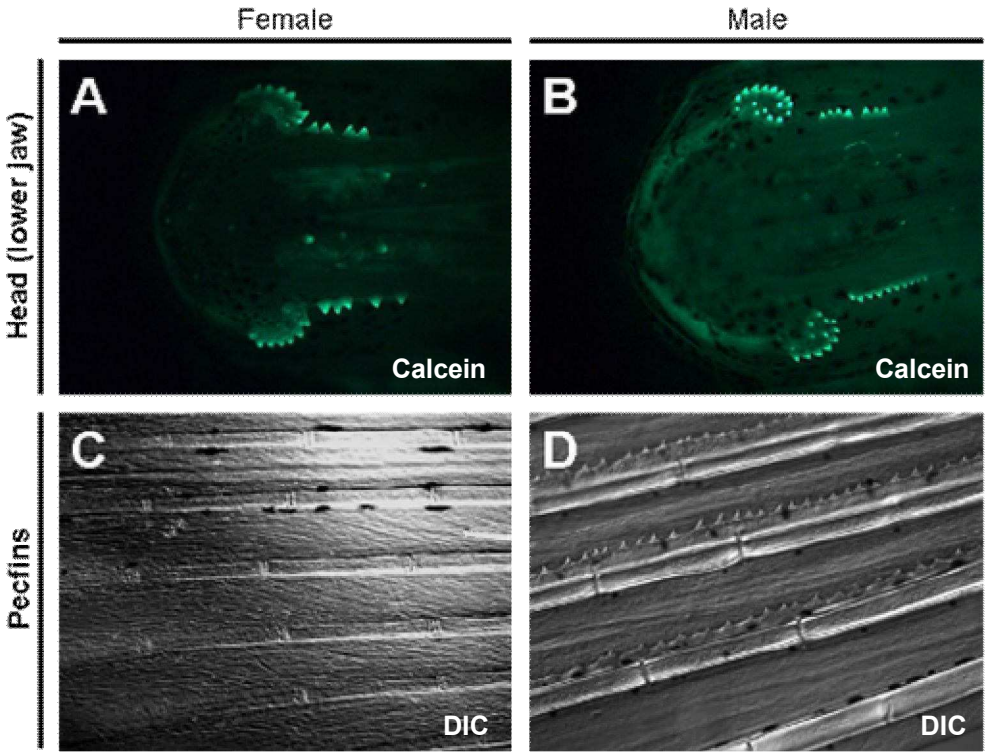


Figure 7: Sexual dimorphism of breeding tubercles in zebrafish. Males and females have the same distribution of tubercles at the lower jaw (A/B), but only males have additional tubercles at the dorsal side of the pecfins (C/D). A/B: calcein staining; C/D: DIC images

Cells of the regular superficial layer got lost in tubercles and cells with unique features were exposed at the outer surface

To analyse the appearance of breeding tubercles and the cap layer in particular, scanning electron microscopy (SEM) was used to compare the epidermal surface of regular epidermis with the surface of breeding tubercles. Adult PFA fixed specimens were prepared either by cryo-fixation or critical-point drying (CPD) technique. All scanning electron microscopy was done at the Central Microscopy Unit of

the Max Planck Institute for Plant Breeding Research in Cologne, with the help of Dr. Elmon Schmelzer and Rainer Franzen.

Generally the outer surface of zebrafish was characterized by hexagonal cells with the typical microridge pattern of superficial cells, as shown in **figures 8A & 8B**. Using lower magnifications, the localisation pattern of breeding tubercles could be confirmed as described before. Tubercles were found at the lower jaw of all specimens (**figure 8C**), and on the fin rays of male pecfins (**figure 8E**).

Using higher magnifications (6000-8000x), the outer surface appeared strikingly different at the breeding tubercles compared to the surrounding epidermis. Again the same results were found for the jaw tubercles and the pectoral fin tubercles. **Figures 8D & 8F** show magnifications of the border region between regular epidermis and jaw tubercles or pecfin tubercles. In the lower part of both images, the characteristic microridge pattern of superficial cells can be seen, as the tubercles were surrounded by regular epidermis. At the base of the tubercle, cell borders were still visible, however the microridges were dramatically reduced (or completely absent) and only bleb-like structures remained. At the tip of the tubercle, in the upper part of **figures 8D & 8F**, the cell surface appeared completely flat and smooth. Microridges were completely absent and cell borders only hardly detectable. These findings led to the suggestion that the smooth cells at the tip of tubercles might have distinct features compared to the regular superficial cells, whereas the cells with reduced microridges or blebs possibly resemble an intermediate state between superficial and cap cells. As it was reported that superficial cells loose their microridge structure during cell death followed by sloughing [Hawkes 1974], the cap layers of breeding tubercles might be considered as death tissue that will be sloughed as a sheet, similar as in mammalian epidermis. Additionally, this would be in accordance to the pyknotic character of most cap cells.

Often, the tissue was broken at the base of the tubercles in the region of intermediate type cells. This observation was underscored by the often detached cap layer in histological sections, and might indicate fragile or defect cell-cell contacts at these sites.

The intermediate type of superficial cells was found at the base of each and between two spikes, at the head tubercles. Only the tips of each spike showed the flat and smooth appearance (**figures 9A & 9B**). This was even more obvious at the tubercle rows of the pecfins. These samples were prepared with the CPD method leading to better quality of the sample tissue. Despite the smaller appearance of these tubercles, the flat and smooth cells presented only at the tip of the tubercles, whereas all cells at the base and between two tubercles showed the intermediate stage (**figures 9C-9E**). Also between two rows of tubercles, no regular superficial cells with microridges could be detected (**figure 9D**). In these specimens, the cellular outlines were clearly detectable, again arguing against a secreted cuticle. Similar as for the jaw tubercles, cracks appeared only at the base of the tubercle in the region of intermediate superficial cells, but never at the tip (**figures 9E & 9F**).

Overall, these results indicated a clear difference of tubercle cap cells compared to regular superficial cells. Probably, the cap layer might be a modification of superficial cells or resemble a distinct cell type with possibly a different developmental origin. As the results of various histological methods indicated

frequently that the cap layer detaches from the layers underneath, and additionally cracks around the tubercles were frequently seen by SEM, one might assume that the cells of the outer layer get lost in the area of breeding tubercles. The intermediate stage between cap cells and superficial cells could only be seen in TEM images but never in any histological methods, where the superficial layer between tubercles always stained in the same way as the cap cells at the tip of the tubercle. Therefore, the intermediate stage cells were considered as a part of the cap layer.

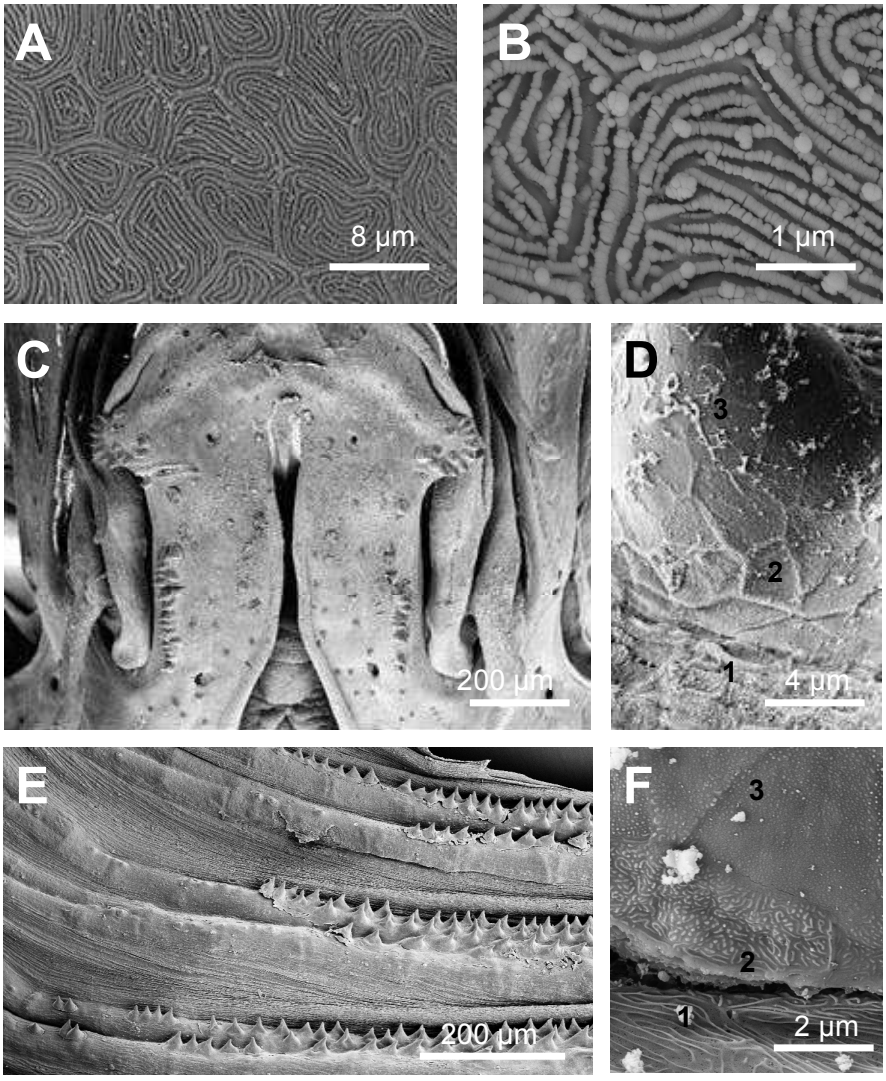


Figure 8: Cap cells of breeding tubercles lack the typical microridge structure of superficial cells. (A/B) Regular epidermis of zebrafish. The outer surface consists of hexagonal cells with the characteristic microridge pattern. (C) Overview of the tubercle distribution at the lower jaw. The disk of tubercles and the posterior row of single tubercle can be seen on both sides of the jaw. (D) Cell surface of cap cells. Around the tubercle, the superficial cells present with microridges (regular superficial layer - 1). At the base of the tubercle, the microridges get lost and the cells obtain a surface covered by bleb-like structures (intermediate stage - 2). The cells at the tip of the tubercle appear completely flat (cap cell – 3). (E) Breeding tubercles on top of the fin rays on the dorsal side of pectoral fins. (F) High magnification of a breeding tubercle of the pectoral fin. The same three stages as described above (superficial cells, intermediate cells & cap cells) can be seen at pecfin tubercles as well.

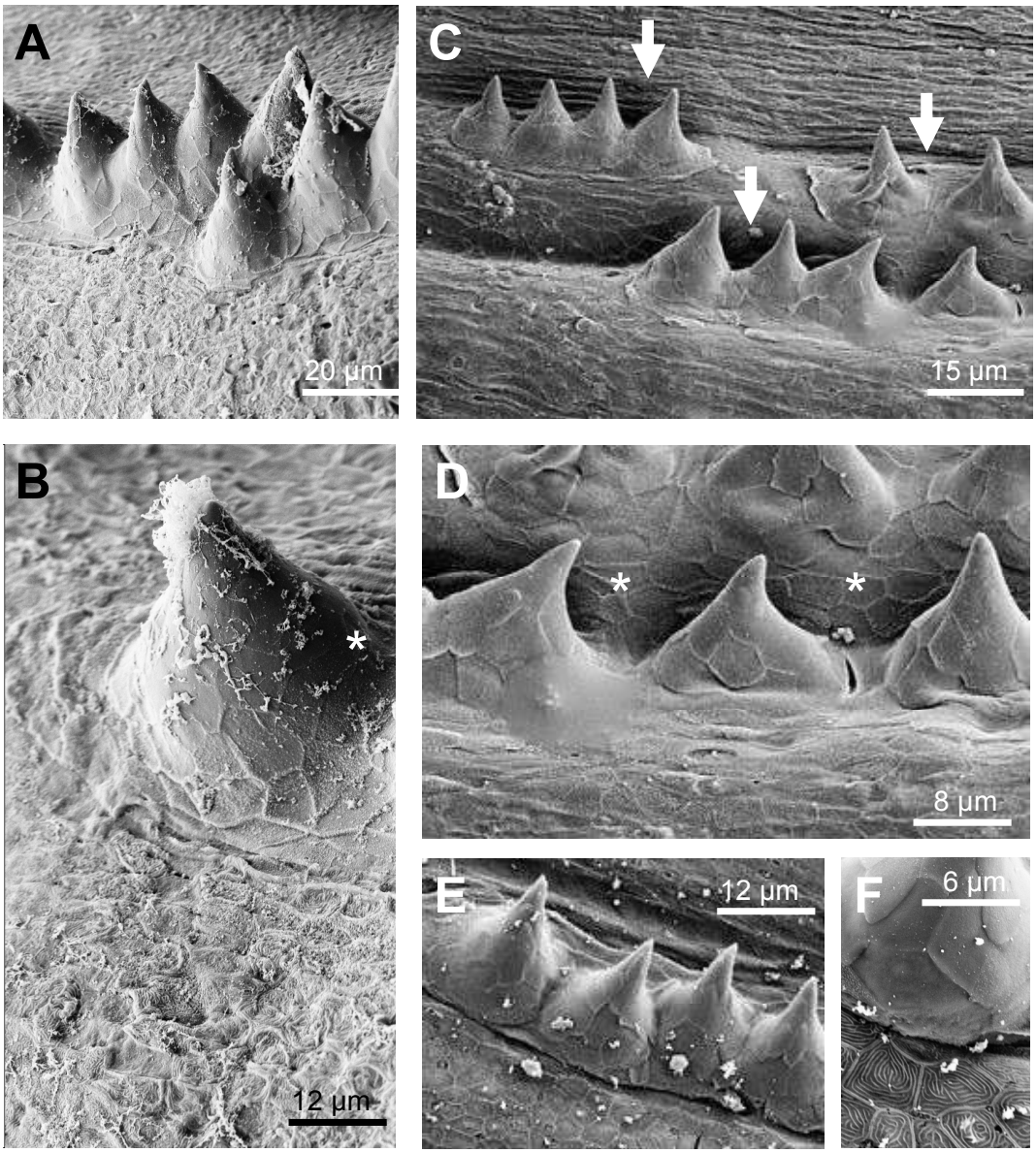


Figure 9: Different types of cell surfaces of breeding tubercles. (A/B) Low magnification of tubercles of the jaw and high magnification of the bridge between two head tubercels of the lower jaw row (asterisk). All cells between the tips of the tubercles display the intermediate phenotype, whereas microridges are not seen in this area. (C) Rows of pectoral fin tubercles on top of the fin rays. Whereas the surface between the fin rays is filled by regular superficial cells, the area between the tubercle tips is filled always with intermediate stage cells (white arrows). (D) If there is more than one row of tubercles on top of a single fin ray, the space between the rows also only consists of internediate stage cells and never of regular superficial layer cells (asterisk). (E) Cracks in the tissue were seen only in the region of the intermediate cell type but never in the surrounding epidermis or at the tip of the tubercles. (F) Higher magnification of the crack region, which is localized in the region of the intermediate type cells.

To further evaluate the difference between cap cells and regular superficial cells, cross-sections of heads and pecfins of adult fish of the transgenic line *krt4::gfp* were performed. In this line, green fluorescent protein (GFP) is driven by the *krt4* promoter and despite controversial *in situ* hybridization data of *krt4* expression (formerly named keratin 8 at ZFIN) [Imboden et al. 1997], fluorescence is detected only in cells of the superficial layer during all larval (data not shown) and adult stages (**figures 10A & 10B**). In contrast to the whole fish which was covered by a fluorescent superficial layer (indicating that the *krt4* promoter is active in this layer), the outermost layer of breeding

tubercles, the cap layers, were always GFP negative (**figures 10A, 10C & 10D**). This finding could also be reconfirmed for pectoral fin tubercles (**figure 10E**). Additionally, Manuel Metzger in our laboratory performed immunostaining with an anti-S100 antibody on adult zebrafish pecfin cross - sections. While the antibody stained all regular superficial cells, staining was absent in the cap cell layer of tubercles. **Figure 10F** was kindly allocated by Manuel Metzger to complement the results described before.

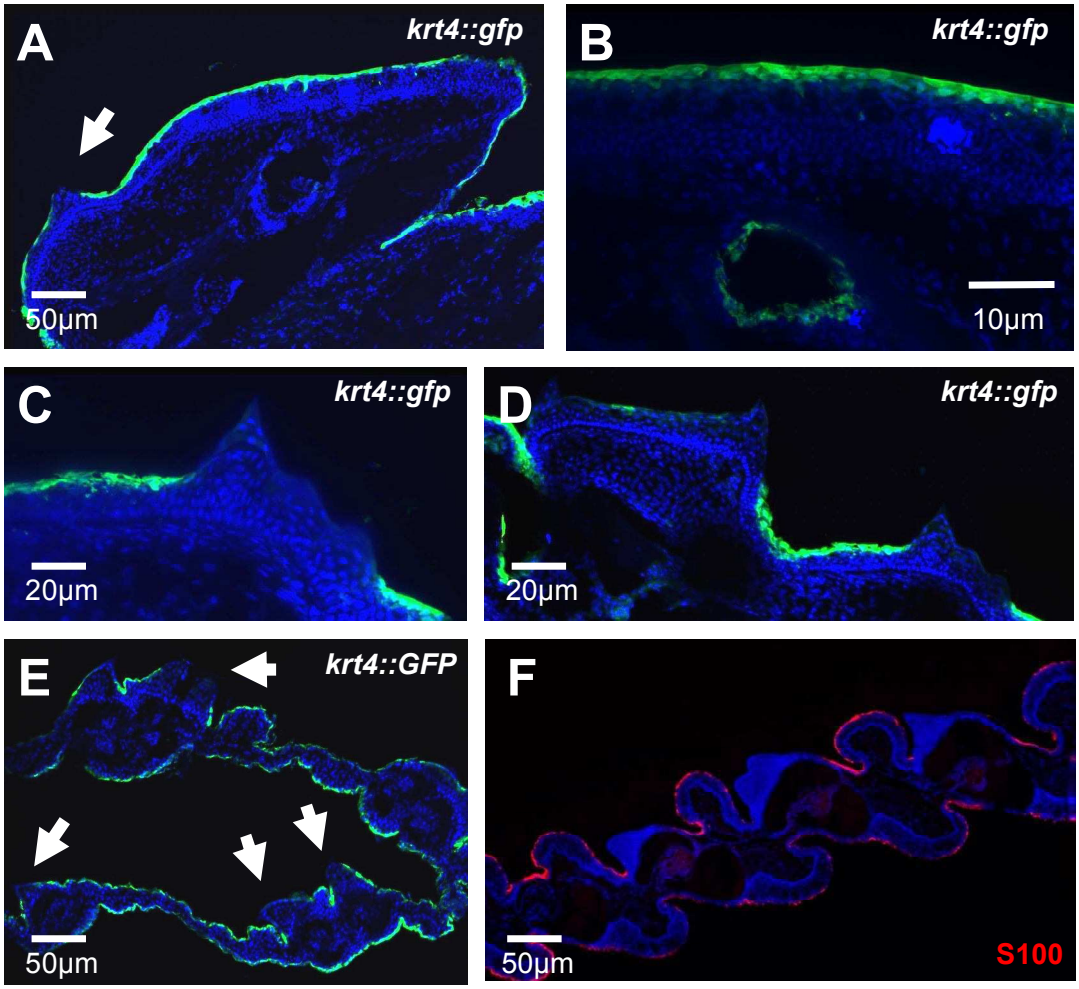


Figure 10: Marker of regular superficial layer are absent in the cap layer of breeding tubercles. All images except F depict cross sections of the transgenic line *krt4::gfp* that expresses GFP only in cells of the superficial layer. (A) Section of the anterior head region. The complete superficial layer is GFP positive, whereas the marker is gone at the small tubercle (arrow). (B) High magnification of head epidermis. Only the superficial layer is marked by GFP. (C) Magnification of a breeding tubercle of the lower jaw row. The superficial layer displays GFP fluorescence around the tubercle. However,the cap layer is clearly GFP negative. (D) Also more complex tubercle structures show the same pattern. Whereas the cap layer of the single tubercles are always GFP negitive, the area between the tubercles is still GFP positive. (E) The absence of GFP in the transgenic line described for the head tubercles can also be seen at the pecfin tubercles. Arrows point to GFP negative cap layers. (F) Also other markers for superficial cells are not detectable in cap layer cells. Immunostaining for S100 which is present in the whole superficial layer, is absent in tubercle cap layers (provided by M. Metzger).

In accordance with the TEM images, the results presented above clearly indicate crucial differences between cells of the cap layer and the superficial layer of regular epidermis. All tested marker for the superficial layer were gone in the cap layer of breeding tubercles. We therefore argued that the superficial layer is lost in breeding tubercles and replaced by cells of the cap layer.

The cap layer of breeding tubercles is shed off indicating the need for a regeneration process in breeding tubercles

As described before, cap layer cells might be sloughed eventually indicating renewal or a regeneration process in breeding tubercles. Indeed, remnants of tubercle cap layers could often be found at the bottom of mating boxes after setting up mating pairs. Whereas long stripes of pecfin tubercles could be detected regularly, complete head tubercle plates were only scarcely found (**figure 11**). Again, DAPI staining clearly demonstrated the cellular character of the sloughed cap layer (**figures 11A, 11D** and the magnification **figure 11C**). Long pieces of sometimes several rows of tubercles still connected to each other, indicated that not only the flat cells at the tip of the tubercles are shed but also the cells of the intermediate type. As already mentioned, intermediate type cells were considered to have the same properties than the cap layer.

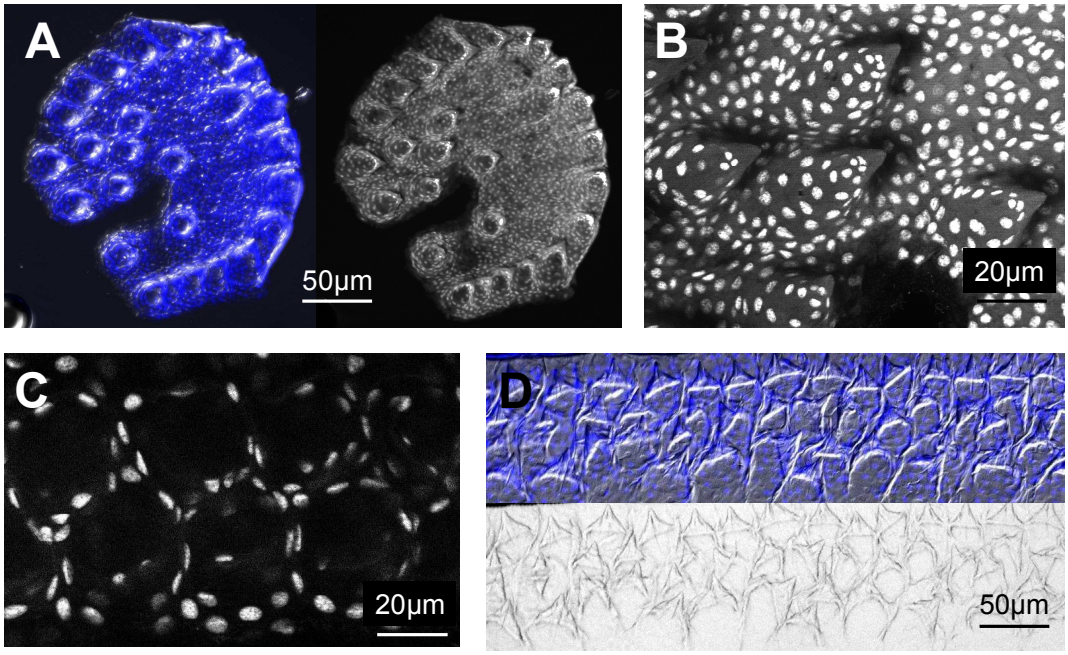


Figure 11: Cap cells of breeding tubercles are sloughed as a whole sheet. (A) A complete plate with tubercles of the head found after mating. DAPI staining clearly demonstrates the cellular character of the shed off cap layer. (B) Magnification of spikes in A. (C) Single plain of a Z-stack of a tubercle spike from figure A. The shed tubercles are hollow demostrating that indeed only the cap layer is shed. (D) Several rows of shed pectoral fin tubercles still connected to each other and stained with DAPI.

The sloughing process of larger parts of the cap layer also led to the conclusion that cell-cell contacts did not loose their functionality between the cells of the cap layer, in contrast to contacts between the cap cells and the cells of the layer underneath.

As it was not possible to embed these cap layer samples for subsequent sectioning, the ApoTome (Zeiss) function of our microscope was used to obtain "confocal-like" plains of a Z-stack. As shown in **figure 11D**, the shed tubercles were hollow structures and the wall of the structure was only one (sometimes two) cell layer thick. This demonstrated that indeed only the cap layer was shed off, whereas all lower layers of the tubercle remained intact, and is in accordance with the healthy appearance of the nuclei of all cells underneath the cap. These findings led to the questions how

these tubercles develop in zebrafish and which mechanisms are responsible for the final shedding process.

Regeneration experiments done by Manuel Metzger in our laboratory indicated that shedding of the cap layer of tubercle happens quite frequently in zebrafish. However the time of regeneration (until a new cap has developed after shedding) was very variable. Pairs of fish were analysed once per day via calcein staining over two month followed by closer evaluation of the head tubercle region each day. All fish had shed at least parts of their tubercle structures two or three times during that period. However, the regeneration period was very variable ranging from five days to up to two weeks, sometimes also in the same fish (Manuel Metzger, unpublished data).

Tubercles develop prior to sexual maturity in a very rapid process

Larval zebrafish do not show any tubercle structures (data not shown). To determine the onset of tubercle development, larval to juvenile stages of zebrafish were observed using a stereomicroscope and by hematoxylin/eosin staining subsequent to paraffin embedding and sectioning. The first small tubercles could be found not earlier than approximately 30 dpf only by histology (data not shown). At this time tubercles could not be seen under the stereomicroscope at all. Overall, onset and size of the first tubercles was very variable. To identify the exact onset of tubercle formation and to analyse the process of development, batches of juveniles were fixed at 21, 24, 28, 31, 35 and 38 dpf, and the standard length (SL) of the individuals was measured. According to the literature, the standard length is a better indicator for juvenile stages of development than dpf [Parichy et al. 2009]. **Table 3** demonstrates that the fish were in the appropriate range according to this staging. Afterwards, the occurrence of tubercles was evaluated by scanning electron microscopy.

Table 3 Length of fish used for scanning electron microscopy

Age	21 dpf	24 dpf	28 dpf	31 dpf	35 dpf	38 dpf
SL [mm]	6.5	7	9	10	12	13
	6	7	9	10	12,5	12,5
	6.5	7	8	11	9	12
	6	6.5	8	10	8,5	12
	6	7	7,5	10	8	10,5
	6.5	6.5	8,5		10	
	5.5	7	9		12,5	
	6	7	8,5		11,5	
	6	7	9		10	
	6.5	7	8,5		9,5	
Range*	**6-6.5**	**6.5-7.0**	**7.5-9.0**	**10-11**	**10-12**	**11-13**

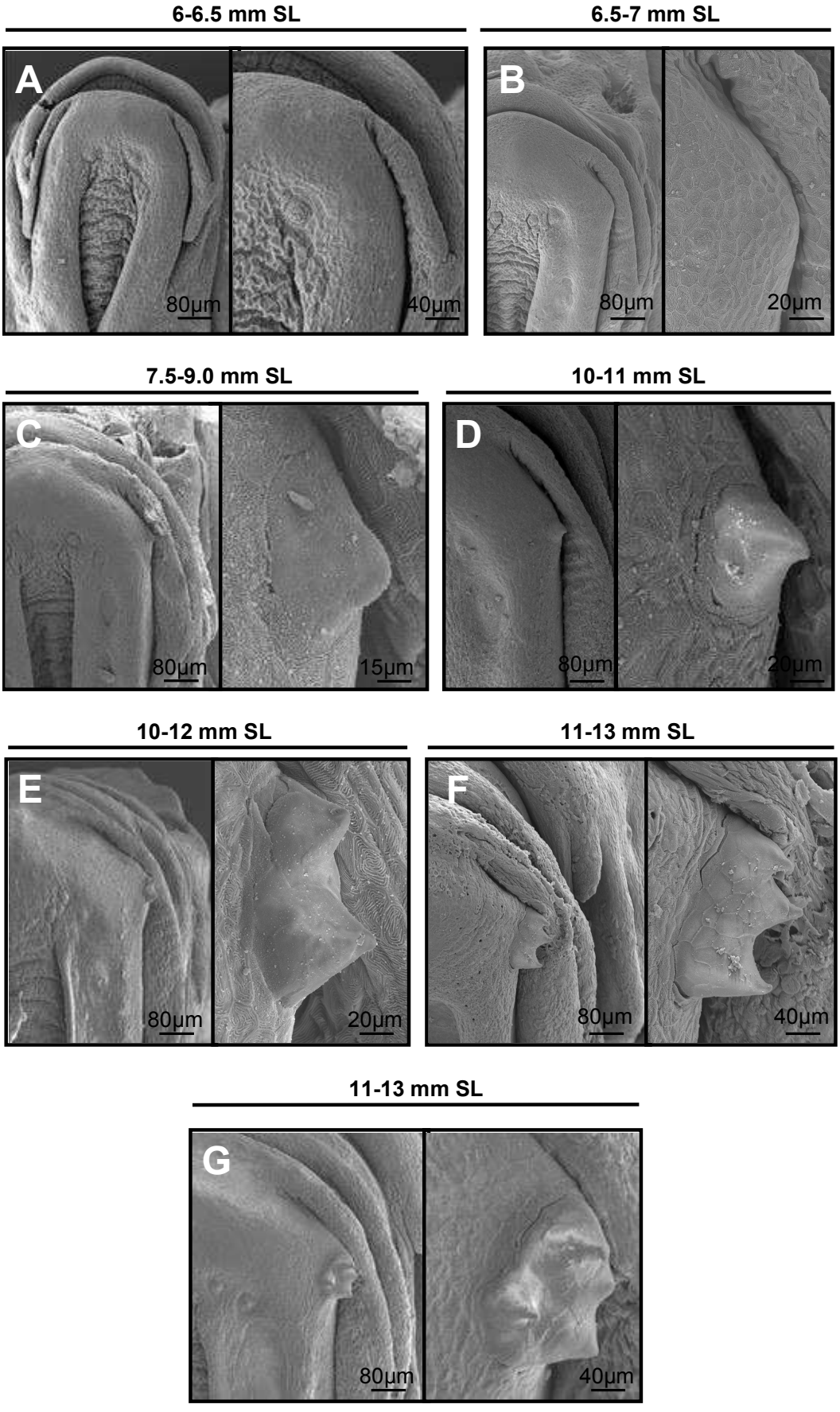
* For some time points the length of individual fish was very variable. In that case, the range was determined that most values were covered.

The smallest juveniles (6-6.5 mm SL) did not present tubercles at all, and the respective position at the lower jaw was completely covered by regular superficial cells with the microridges (**figure 12A**).

Soon later (SL 6.5-7.0 mm & SL 7.5-9.0 mm), a small bulge could be detected at the edge of the lower jaw near the developing barble. This swelling was still covered completely by regular superficial cells (**figure 12B**). The same batch already contained fish were the first tubercle had started to from on top of the bulge and developed the cap layer, indicating rapid progression of tubercle formation (**figure 12C**). However, the initial shedding of superficial cells could never be detected. The freshly developed cap cells were of completely flat appearance but cell borders could still be detected.
At 10-11 mm SL, the tubercle had grown in size and appeared more steeply with a smooth surface (**figure 12D**). In the following period, a second and a third tubercle formed rapidly in a row (**figures 12E & 12F**), until finally a three-dimensional superstructure, the tubercle plate, had developed (**figure 12G**).
Figure 12H depicts a tubercle (SL 11-13 mm) shortly after partial shedding of the cap layer. The cells of the layer underneath (2nd tier layer) are now exposed to the environment. These cells look clearly different than the cap cells with numerous small aggregates at the surface that might resemble remnants of former cell-cell contacts to the cap layer. Accordingly, shedding of the cap layer seems to start directly after development of the tubercle has been completed and (according to the previously presented results) seems to be maintained during adulthood.

The row of head tubercles caudally of the tubercle plate has still not developed at the described stages. However, tiny hillocks developed (starting at SL 11-13 mm) in regular distances at the locations the row of tubercles will appear later (**figure 12I**). On top of these elevations was always a single cell that appeared similar to the intermediate stage cells at the base of mature tubercles. Even if these elevations shared a lot of similarity with (at that stage already present) taste buds [Hansen et al. 2002], the bleb-like structure of these "core cells" might also indicate the first cap cell of a small newly developed tubercle. However, later stages of tubercle development were not observed in this study.

Pecfins of males of the *bactin2::mGFP* transgenic line (membrane bound GFP) were used to investigate the developmental progress of pectoral fin tubercles. Fixed whole-mount pecfins of different stages were mounted with Mowiol/DAPI and observed directly with a fluorescence microscope including the ApoTome (Zeiss) module.
Regular DIC images revealed that pectoral fin tubercles developed at the same time as the tubercles at the lower jaw. Whereas no tubercles could be detected at SL=7.5 mm, the formation of small characteristic cell aggregates on top of the fin rays could be detected at SL=8.5 mm (**figures 13A & 13B**). At that time, a cap layer has not formed yet. This intermediate stage of development might resemble the formation of the small, more flat tubercles at the same time point at the lower jaw. However, a fully developed cap layer was detected only shortly later (**figure 13C**, SL 9.5 mm).


6-6.5 mm SL
6.5-7 mm SL
A
80µm
40µm
B
80µm
20µm
7.5-9.0 mm SL
10-11 mm SL
C
80µm
15µm
D
80µm
20µm
10-12 mm SL
11-13 mm SL
E
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F
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40µm
11-13 mm SL
G
80µm
40µm

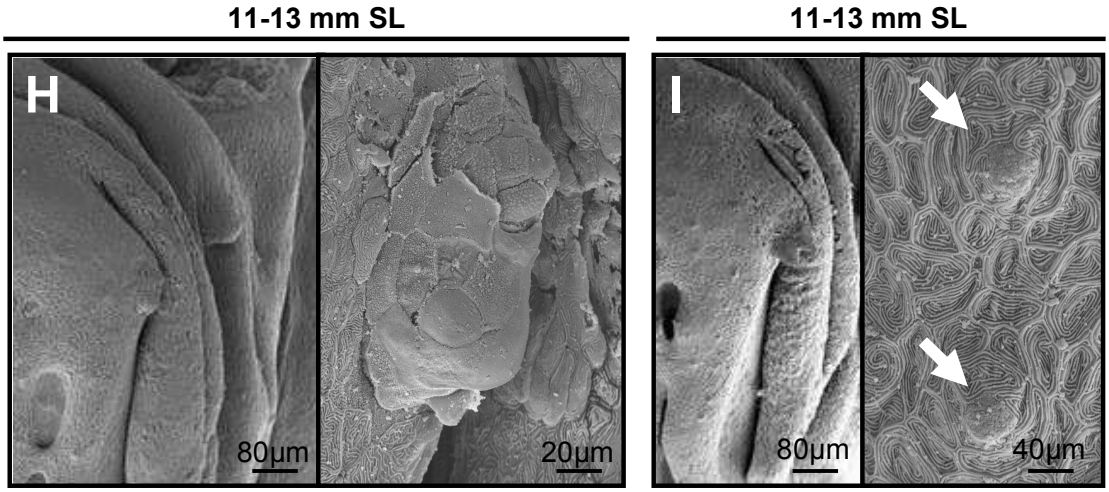


Figure 12: Stages of breeding tubercle development. (A) View of the ventral side of the lower jaw prior to breeding tubercle development. The complete jaw is covered by regular superficial cells (SL 6-6.5 mm). (B) A small bulge has developed at each side of the jaw (6.5-7 mm). The elevation is still covered by regular supericial cells. The tubercle spikes will develop on top of this elevation during the next steps. (C) A first tubercle has developed on top of the initial bulge. Even if the tubercle is not as steep as a mature tubercle, the cap layer seems to be already present. (SL 7.5-9 mm). (D) Maturation of the first tubercle is completed (SL 10-11 mm). (E-G) In the following, more tubercles develop until a three dimensional structure, the tubercle plate has developed (SL 10-13 mm). (H) Shedding seems follow of tubercle development directly. Half of the cap layer has been torn away and the underlying second tire becomes visible (SL 11-13 mm). (I) The dorsal row of tubercles at the head has still not been developed, but small elevations already occured on top of which one cell seems to display the intermediate phenotype of tubercles (arrows) (11-13 mm).

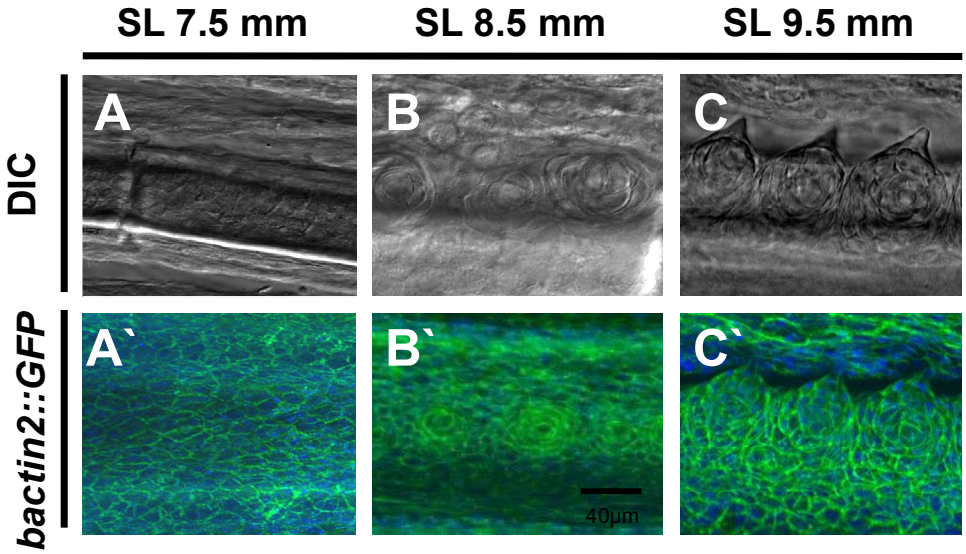


Figure 13: Pectoral fin tubercle development. Development of the tubercles on the dorsal side of the pectoral fins. DIC images and the corresponding fluorescent images of the *bactin2:GFP* transgenic line are shown. At 7.5 mm SL no tubercle structures are detectable on top of the fin rays (A & A‘). Later, at 8.5 mm SL small cell aggregations have been formed that are still not covered by a cap layer (B & B‘). The cap layer has been formed only shortly later at 9.5 mm SL (C & C‘). The transgenic line demonstrates the cellular character of the tubercle and the cap layer in particular at all stages of the developmental process.

By exploiting fluorescence microscopy, cell borders of epidermal cells were clearly be detectable in *bactin2::GFP* transgenic fish, in which membrane bound GFP is expressed under the control of a ubiquitous active *β-actin2* promoter. Before tubercle development, the fin rays are covered by regular

epidermal cells. At 8.5 mm, the above mentioned cell aggregates could also be detected with clearly outlined cell borders. Even after development of the cap layer, single cells still could be distinguished in the transgenic line, indicating that despite the smooth appearance of the cap in SEM images as well as in the DIC figures, the cap still consisted of individual cells (**figures 13A'-13C'**).

The SEM images of juvenile zebrafish clearly demonstrated that the regions where the tubercles will develop later are initially covered by regular superficial cells. However, all tested superficial maker were not detected in mature cap layers, indicating that the superficial layer has been sloughed. To evaluate when this loss of superficial layer markers occurs and if it correlates with the formation of the cap layer, fluorescence microscopy of whole-mount pecfins and heads of *krt4::gfp* transgenics was performed during the above described developmental period.
Initially the pectoral fins were covered by regular hexagonally shaped superficial cells. The small cell aggregates described before were also detected in this line (data not shown). At the beginning, tubercles were still covered by GFP positive cells, indicating their regular superficial cell character.
Shortly later GFP fluorescence ceased in single cells of the tubercles (**figure 14**). This loss of GFP fluorescence was detected at SL=9 mm around the same time the cap layer has been shown to form.

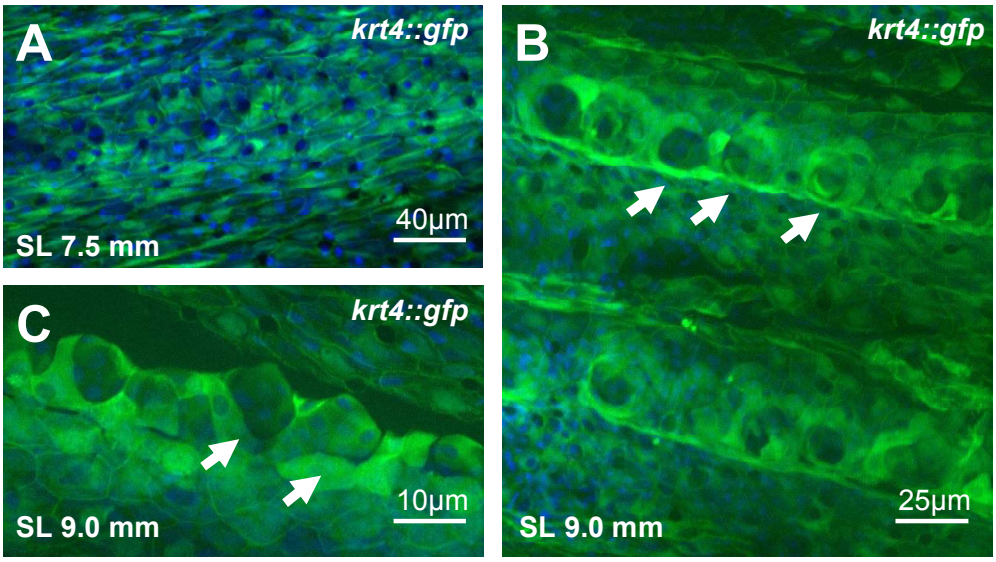


Figure 14: Breeding tubercles loose the superficial layer cells when the cap develops. Different stages of breeding tubercle development of the pectoral fin in the transgenic line *krt4::GFP*. (A) Superficial layer of the pectoral fin prior to tubercle development. (B/C) GFP levels cease at the time cap layer development occurs. However, this step does not occure exactly at the same time in all tubercles in a row (white arrows).

The same results were also found for head tubercles of *krt4::GFP* fish. Similar as for the pectoral fins, GFP fluorescence ceased in the developing tubercles when formation of the cap layer had started. However, the small cell aggregates (as an intermediate stage of tubercle development at the pectoral fins) were not found at the head. The initial bulge, which was still covered by regular superficial layer cells, was also detected in the *krt4::GFP* transgenic line. The first small tubercles developed rather early on top of this elevation and coincided with the loss of GFP superficial cells (**figures 15A-E**).

Altogether, tubercle development of jaw tubercles as well as pectoral fin tubercles occurred between SL 8.5 mm and 9.5 mm in a very rapid process. The loss of superficial markers, most likely due to shedding of the superficial layer in an initial shedding cycle happened simultaneously to the development of the first cap layer.

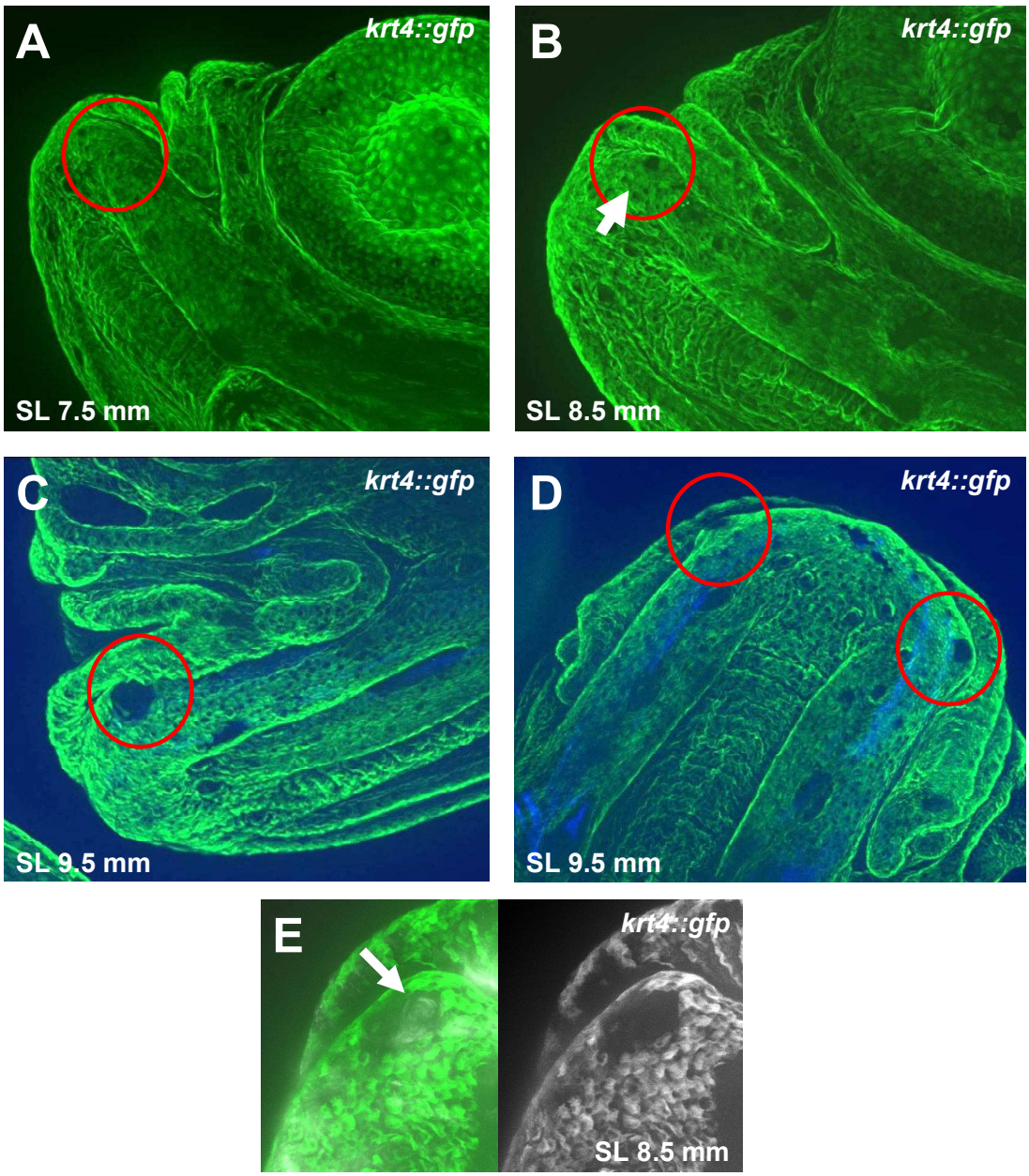


Figure 15: Loss of periderm coincides with the development of the cap layer of head tubercles. Whole-mount images of *krt4::GFP* transgenic fish at different stages of head tubercle development are shown. The site of tubercle formation is marked by red circles. (A) At 7.5 mm SL, breeding tubercles still have not developed. The lower jaw is covered by regular GFP expressing superficial cells (lateral view). (B) at 8.5 mm SL, a bulge has formed on the lateral side of the jaw, which is still covered by superficial cells. However, in a small region, the GFP fluorescence is already lost at this stage (white arrow). (C) At 9.5 mm SL, the region where GFP florescence is lost has increased greatly in size. (D) At the same stage, ventral view shows that this process had occured on both sides of the jaw but with slight timely variations. THe loss of GFP seems to be greater on the left than on the right side. (E) Magnification of the tubercle region shown in B. The left image is an overlay of the bright field channel indicating the formation of the first mature tubercle (arrow) and GFP channel. The left figure is the GFP channel alone. The region, where GFP is gone correlates perfectly with the region, where the first cap layer becomes visible.

The superficial layer of adult zebrafish epidermis is gradually replaced by basal derivatives whereas the cells of the cap layer develop exclusively from the basal/intermediate layer

The origin of the superficial cells in zebrafish epidermis is still not completely understood. The EVL of embryonic stages shows many structural similarities with periderm cells of mouse embryos like flattened cell shape, tight junction complexes and microridge structures [LeGuellec et al. 2004], but does not derive from basal keratinocytes as it is the case in mammalian epidermis. Initially it was suggested that the enveloping layer gets replaced after embryonic development [Kimmel 1990]. However, newer data indicate that the EVL is at least maintained until larval stages [Fukazawa 2010]. In the *krt4::gfp* line, GFP is expressed in superficial cells throughout the complete lifetime. Additionally, the appearance of superficial layer (cell shape, microridges) cells is very similar to embryonic EVL cells. This might indicate that the embryonic EVL is maintained throughout metamorphosis or even adulthood. To test whether the EVL is replaced by cells coming from the intermediate layer or maintains itself autonomously throughout larval development and adulthood, we performed lineage tracing experiments using a tamoxifen-inducible Cre/lox system [Kaslin et al. 2009] to specifically label embryonic EVL cells by a *krt4* driver [Gong et al. 2002] or basal cells by a *krt19* driver. As we already demonstrated that the superficial layer is lost in breeding tubercles and the outermost layer seemed to develop by a differentiation process in the tubercles giving rise to cap layer cells with distinct functions compared to the regular superficial layer, we wanted to use our lineage tracing system to compare the situation in regular epidermis to the breeding tubercles. According to our data we hypothesized that the cap layer may develop exclusively from intermediate or basal cells.

The *krt19* promoter construct was kindly provided by Tom Carney (Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore) All other transgenic lines of this experiment, were established by Rebecca Richardson and Philipp Knyphausen by BAC recombination in our laboratory.

Initially, we demonstrated the specificity of the mentioned promoter fragments with five day old *krt19::dtomato; krt4::gfp* double transgenics. After the larvae were sacrificed they were directly used for cryo-sectioning. Immunostaining was not performed.

During embryonic and early larval stages, the epidermis of zebrafish consists of only two layers: the basal layer and the EVL. **Figure 16A** demonstrates that that the *krt19* promoter was active only in basal cells (shown by red fluorescent cells). This domain did not overlap with the EVL cells labelled by green fluorescence due to the *krt4* promoter fragment.

In the following, we crossed *krt4::gfp; bactin2::loxP-STOP-loxP-dsred* double transgenics with identified carriers of the *krt19::CreERt2*, and the *krt4::CreERt2* driver line respectively, to obtain triple transgenics. In these lines, the inducible version of Cre recombinase would be expressed only in those cells where the promoter of the driver constructs are active. Additionally, Cre recombinase was activated by tamoxifen from 1 dpf to 5 dpf. At that time the epidermis of zebrafish larvae is still bilayered. Therefore, activation of the Cre recombinase occurred only during a developmental period when all epidermal cells which are not EVL cells are basal cells and no other cells exist. The timely

restricted treatment prior to formation of the intermediate layer ensured that all epidermal cells during these larval stages could be targeted. As labelling occurs by recombination of DNA between the two loxP sites, the STOP cassette gets lost only in labelled cells and allows the expression of the fluorophore DSRED by the ubiquitous *β-actin2* promoter, which is later additionally the case in all cells that derive from these initially labelled cells. Activity of the Cre recombinase is therefore controlled spatially and timely.
The tamoxifen treated double transgenics were sacrificed after approximately two months of development when the epidermis has become multilayered and used directly for cryo-sectioning. Of all triple transgenic lines mentioned before sections of the head, the trunk and the pectoral fin (in case of males) were performed.

Unexpectedly, the two distinct domains of embryonic epidermis were not completely separated anymore at the observed stage (two month of age). Sections of treated *krt19::CreERt2; krt4::gfp; bactin2::loxP-STOP-loxP-dsred* triple transgenics revealed DSRED labelled cell clones predominantly in the basal and intermediate layer. But DSRED positive cells were also frequently detected in the superficial layer and co-labelled by GFP (**figures 16B-16D**). This result was found for sections of the trunk, the fins and the head, and would clearly indicate that at later stages of development, the intermediate layer contributes to the maintenance of the superficial layer. Possibly, the embryonic EVL will be replayed completely in the course of time.
Importantly, the cells of the superficial layer that derived from the basal layer expressed the same marker genes (GFP in the *krt4::gfp* line) as the derivatives of the EVL (GFP positive but DSRED negative cells) and had adopted the same characteristics as for example cell shape or microridges, indicating equal properties and functions.
Interestingly, the basal cells displayed very bright intensity of DSRED fluorescence, whereas the intensity ceased throughout the different cell layers of the intermediate layer. As DSRED is driven by the ubiquitous *bactin2* promoter, this might point to a lower activity of the promoter in the intermediate layer compared to the basal layer.
The breeding tubercles were completely labelled by DSRED positive cells, indicating that they are indeed exclusively derivatives of the basal epidermis. In accordance with previous results, GFP positive cells were lost in the cap layer of tubercles (**figure 16E**).
In summary, the superficial layer of the regular epidermis was found to be a mixture of cells originating from the basal layer and from cells that derive from the embryonic EVL. In contrast, the epidermal cells of the breeding tubercles completely derived from basal cells.

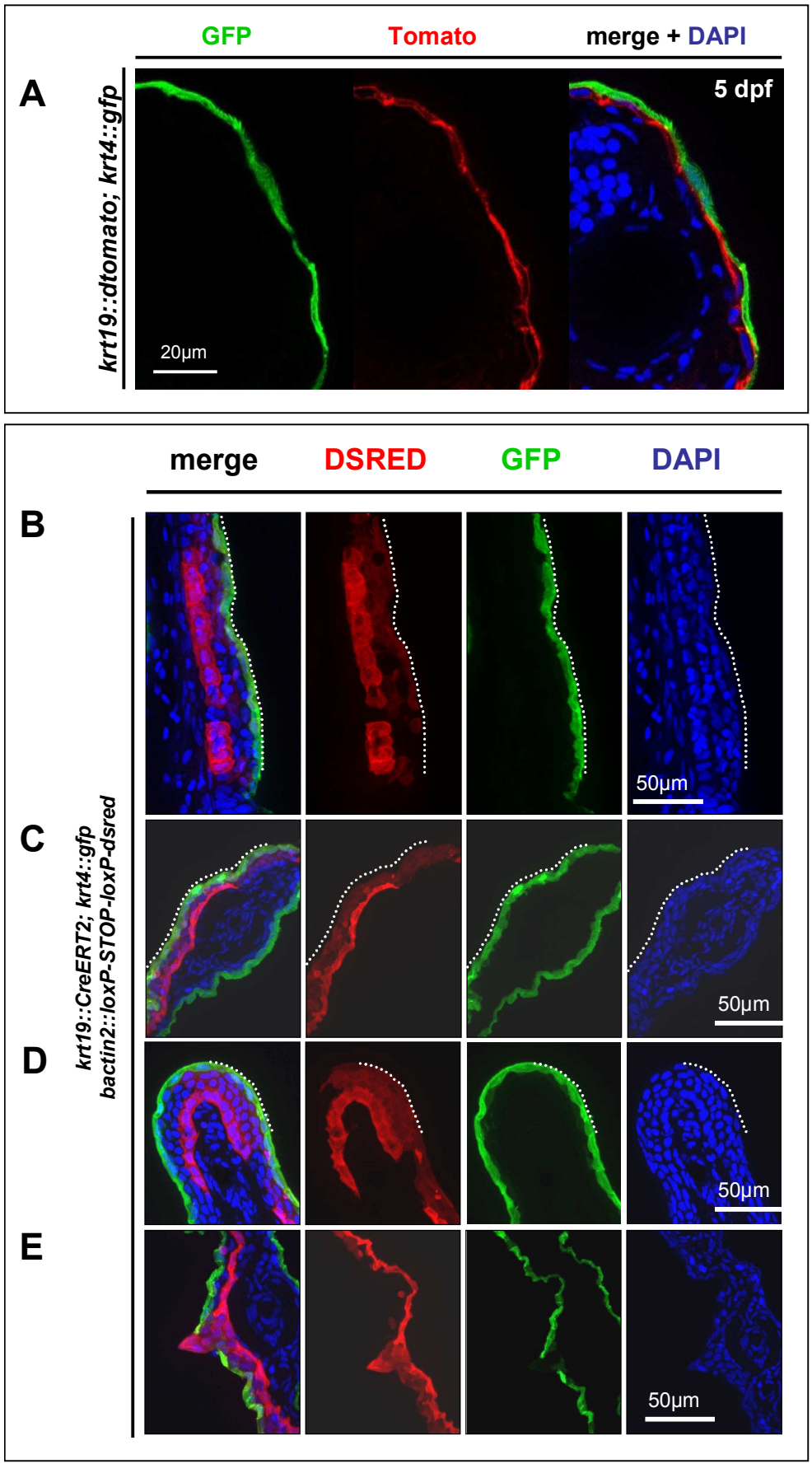
However, this model was complicated by evaluation of the reverse experiment. *krt4::gfp; bactin2::loxP-STOP-loxP-dsred* double transgenics were crossed to the *krt4::CreERt2* driver line. The obtained tripe transgenics were tamoxifen-treated and analysed as described above.
Here, recombination should occur only in cells of the embryonic/larval EVL. We speculated that these DSRED labelled cells should still be part of the superficial layer and thereby be labelled in both, red

and green. This was indeed the case for many superficial cells. However, additionally many cells of the intermediate layer were also found to be labelled by DSRED (**figures 16F-16I**). In rare cases also the basal layer was labelled. This would indicate that cells of the superficial layer might also contribute to the intermediate and perhaps even the basal layer of the epidermis. Whole-mount images of younger triple transgenic fish (10 dpf) revealed that already at the time the intermediate layer forms cells leave the superficial layer and contribute to the new intermediate layer (**figure 16J**).
In general, breeding tubercles were not stained in this triple transgenic line due to loss of the superficial layer above the tubercles (data not shown). However, in very rare cases also DSRED labelled cells in the upper layers of tubercles could be detected, and might be explained by cells that moved from the superficial layer downwards to the intermediate layer prior to tubercle formation (**figure 16K**).

In summary, regular fish epidermis seems to become a mixture of cells that derive from the embryonic EVL or from basal epidermal cells. Both types of cells have the same appearance and function in the end. However, if the proportion of replaced superficial cells increases during adulthood, the situation of adult zebrafish epidermis would resemble the situation of embryonic mouse epidermis before cornification has started. There, the multilayered epidermis is still covered by viable periderm cells that originate from the basal layer.
Accordingly, the cells of the cap layer might develop from derivatives of both embryonic layers as well. However in general, they derive exclusively from the basal layer of the epidermis, only if in this region cells from the superficial layer contributed to the basal layer prior to tubercle development, then also cells from the superficial layer might contribute to the tubercle. This means that cells of the basal layer (and possibly also derivatives of the superficial layer) have the property to eventually become cap cells.
It might be concluded that there are two different types of differentiation in adult fish epidermis originating both from the basal layer. One type ends in viable superficial cells and the other in cap cells of the breeding tubercles that are shed regularly. Additionally, our data indicate that not only the superficial layer is a mixture of cells with different origin but the intermediate and perhaps even the basal layer as well.

Breeding tubercles are rich in keratin content with differential keratin expression compared to the surrounding epidermis

In vertebrates, the cytoskeleton of keratinocytes is reinforced by type-I and type-II keratin intermediate filaments that consist of type-I/type-II heterodimers. According to literature, fish keratins are evolutionary very distant from mammalian keratins with an additional excess of type-I keratins compared to type-II [Schaffeld et al 2007]. In contrast to mammals, keratins are not restricted to epithelia in fish, but are sometimes also found in mesenchymal tissues [Conrad et al 1998].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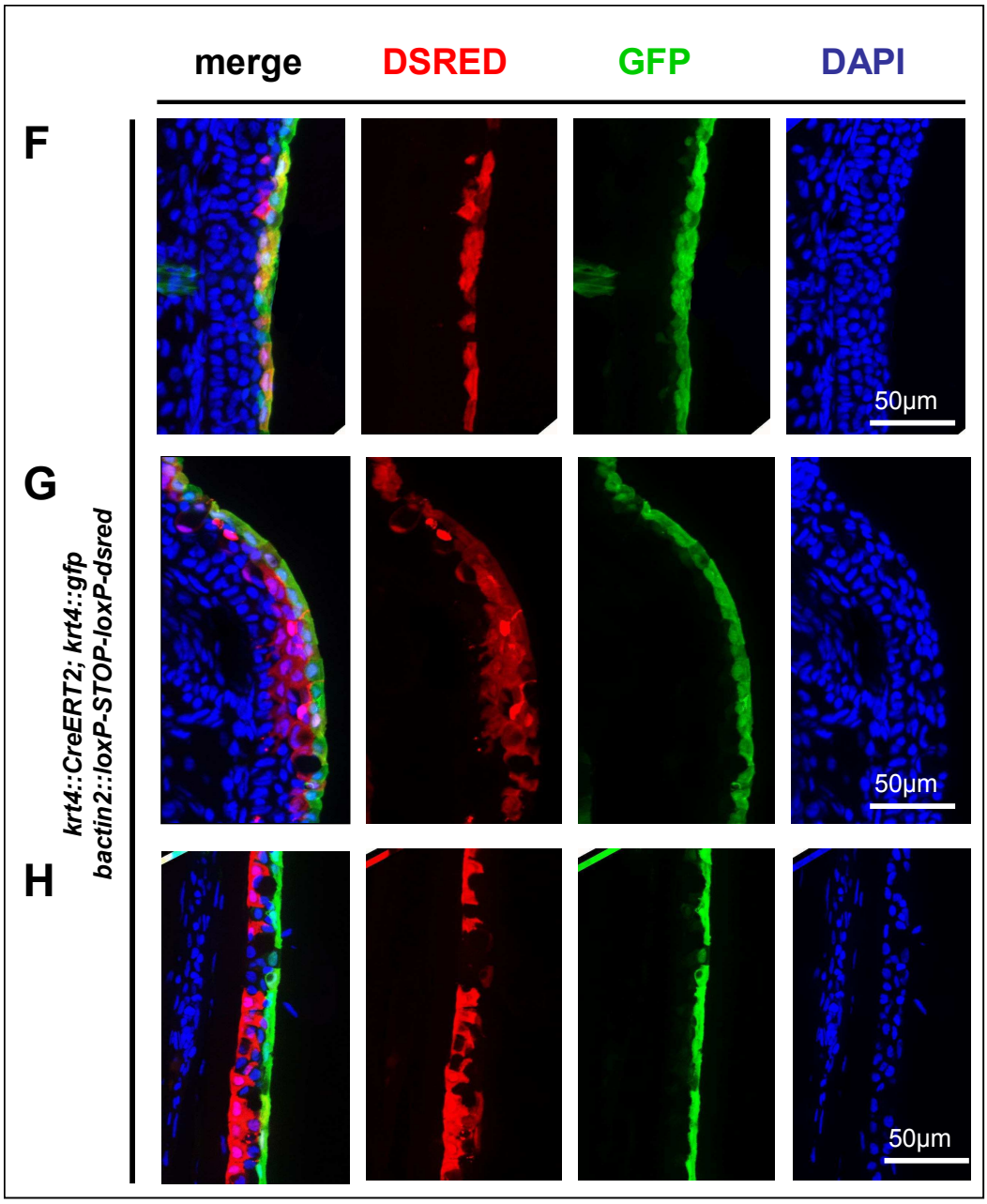
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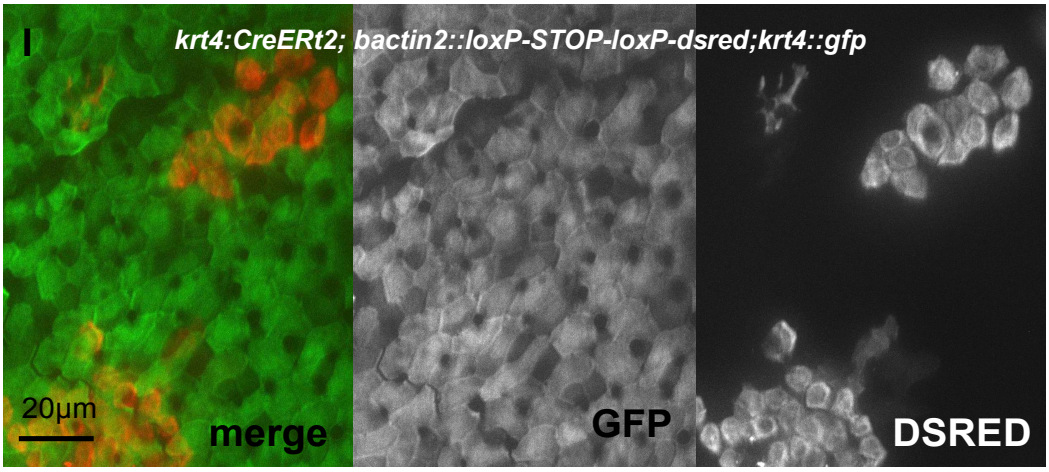
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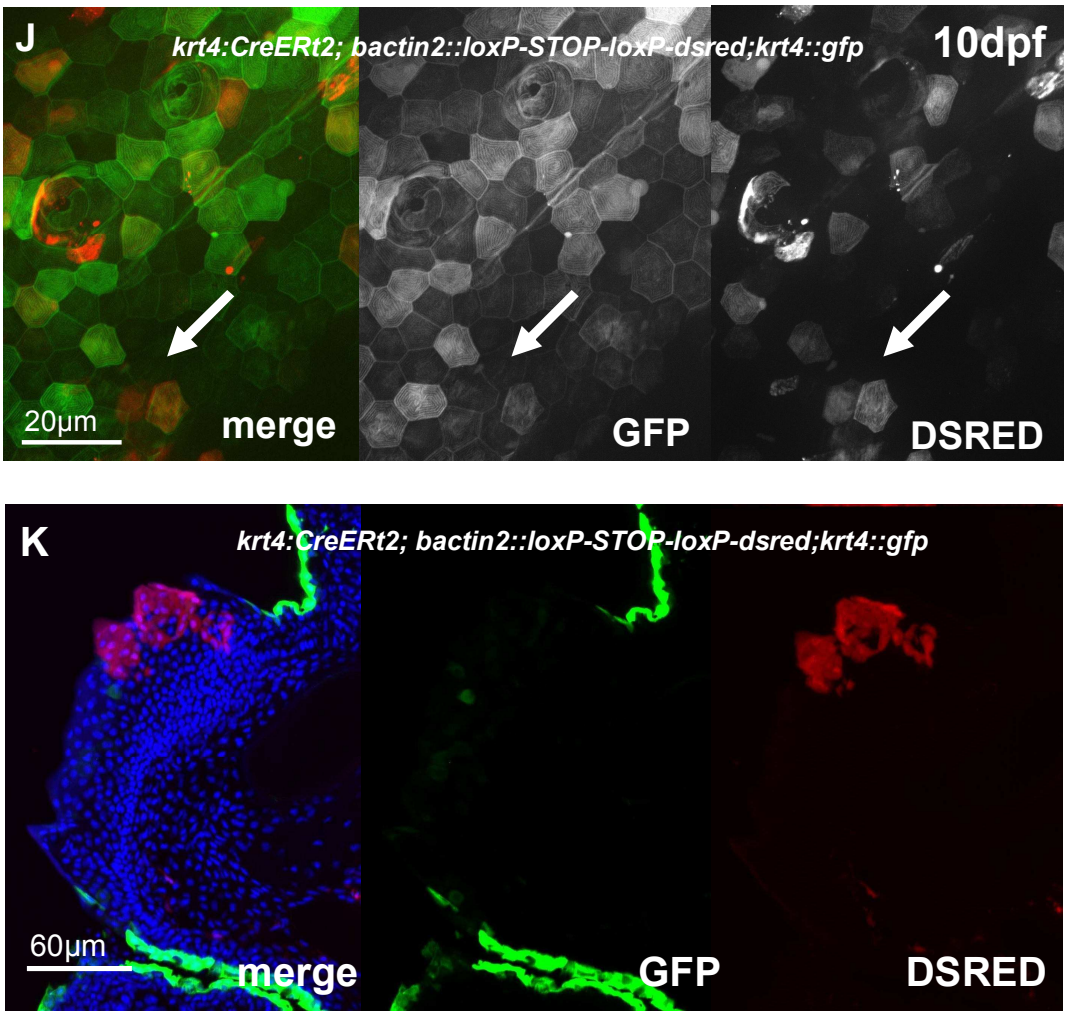


Figure 16: The superficial layer of adult zebrafish is a mixture of cells that derive from the embryonic EVL and from the embryonic basal compartment, whereas the cap layer of breeding tubercles derives from basal cells exclusively. (A) Sections of larval transgenic fish (*krt19::dtomato; krt4::gfp*) sacrificed after 5dpf. The red fluorescent domain (*krt19* driver) does not overlap with the green fluorescent domain (*krt4* driver). (B-E) Sections of adult triple transgenic zebrafish (*krt19::creert2; bactin2::loxP-STOP-loxP-dsred; krt4::gfp*). Tamoxifen treatment was performed from 1dpf until 5dpf. The red fluorescent cells that are derived from embryonic basal cells and the green fluorescent cells (are derived from the embryonic EVL) are not completely separated anymore during adulthood. Breeding tubercles exist completely (including the cap layer) of cells from the embryonic basal layer (sections: B – trunk; C – dorsal fin; D – dorsal fin; E – pectoral fin with breeding tubercle). (F-H) Sections of adult triple transgenic zebrafish (*krt4::creert2; bactin2::loxP-STOP-loxP-dsred; krt4::gfp*). Tamoxifen treatment was performed from 1dpf until 5dpf. The DSRED positive cells are not only found in the superficial layer (F) as initially expected, but also frequently in the intermediate layer (G) and even in the basal layer (H). (I) Z-stack of the epidermis of adult *krt4::creert2; bactin2::loxP-STOP-loxP-dsred; krt4::gfp* triple transgenic adult zebrafish. The DSRED positive clone clearly consists of cells that do not belong to the superficial layer. (J) Already at 10 dpf, shortly after the intermediate layer has formed, some DSRED positive cells in *krt4::creert2; bactin2::loxP-STOP-loxP-dsred; krt4::gfp* triple transgenics are in the layer underneath the superficial layer (white arrow). (J) Most breeding tubercles in 2 month old *krt4::creert2; bactin2::loxP-STOP-loxP-dsred; krt4::gfp* triple transgenics are negative for both fluorophores. However, occationally DSRED positive cells were also found in upper layers of breeding tubercels indicating that cells of the embryonic EVL or larval superficial layer conributed to the basal/intermediate compartment prior to tubercle formation.

However keratins are still a crucial part of fish epidermis, where they form a scaffold that enables the cells to resist against mechanical stress [Coulombe & Bernot 2004]. Especially in mammalian epidermis keratins are also important molecules in the process of keratinization (intensive bundling and crosslinking of keratin filaments) and cornification (differentiation process of keratinocytes including keratinization, membrane alterations and cell death) [Bragulla & Homberger 2009].

We used a polyclonal mouse anti-pan-Keratin antibody (recognizes mouse type-II; keratins 1-8), to study keratin content in breeding tubercles and regular epidermis of zebrafish (**figure 17**). Immunohistochemistry was performed on paraffin- and cryo-sections. The antibody detected low amounts of keratins in all layers of regular zebrafish epidermis (**figure 17A.** However, the levels of keratin seemed to be strongly elevated in the upper layers of the breeding tubercles. **Figures 17B-17D** show that keratin content was greatly increased in the additional layers of the tubercles including the cap layer. However, high amounts of keratin do not necessarily indicate keratinization or cornification but are an important prerequisite.

In mammalian epidermis, differentiation of keratinocytes includes differential expression of certain keratins in the different layers of the epidermis. Specific keratins are needed for specific functions of the cells in certain layers. A "keratin code" like it is described for mammals is not known so far for fish.

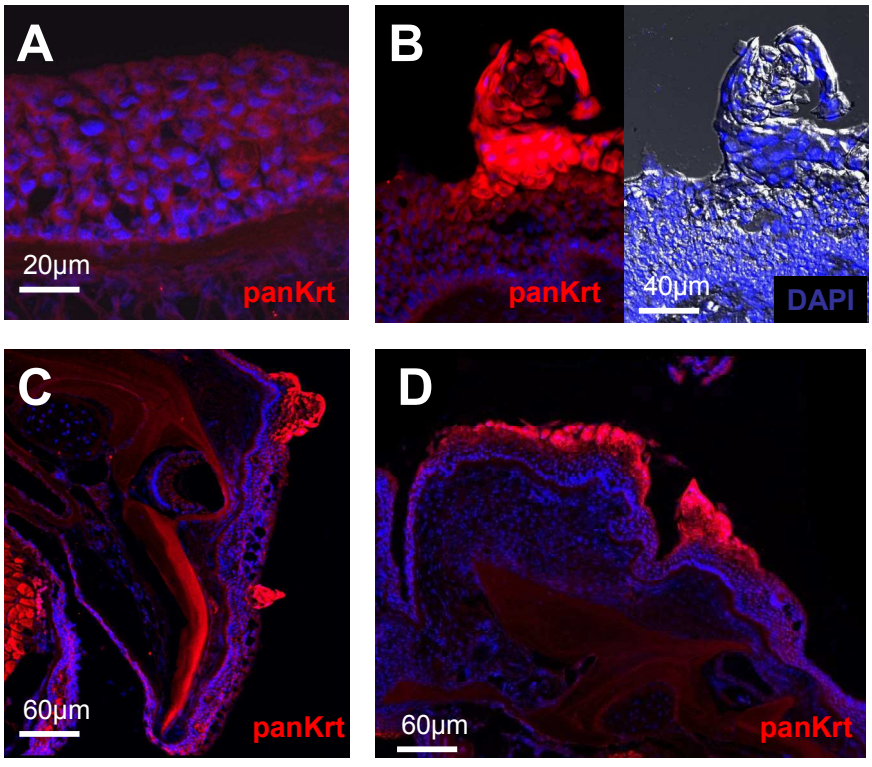


Figure 17: The cellular amount keratin is highly increased in the upper layers and in the cap layer of breeding tubercles. Immunostaining with a pan-Keratin antibody (recognizes Keratin 1-8). (A) Regular head epidermis. Only low amounts of Keratin could be detected. (B) Breeding tubercle with surrounding epidermis. Compared to the surrounding epidermis, keratin content is greatly increased in the upper layers of the breeding tubercle including the cap layer. The left image shows an overlay of the RFP channel visualizing Keratin content and the DAPI channel. The right image depicts bright field togther with DAPI. (C) Low magnification image of the jaw row tubercles. The antibody signal increases greatly towards the outer surface. (D) Low magnification image of the tubercle disc on the head showing the same staining pattern.

The expression pattern of six different zebrafish keratins was analysed in adult fish epidermis by *in situ* hybridization. The expression in regular epidermis was compared to breeding tubercles. The different keratin probes used for this study are listed in **table 4.**

During early larval stages five of the six keratins were expressed overall the epidermis (**figures 18A, 18B & 18D** and data not shown). Cross sections revealed that all these keratins are expressed in the basal and in the superficial layer of the bilayered epidermis at these stages (**figures 18E, 18F** and data not shown). In contrast the sixth keratin, *cytokeratin E7* (*cke7*), had an expression pattern restricted to the tip of the larval finfold and the pecfins (**figure 18C**).

Table 4: Zebrafish keratin RNA probes used for *in situ* hybridization

Keratin	name	Chromosome	Reference	Type
krt4	Keratin 4	6	Gong et al. 1997; Webb et al. 2008	II
krt5	Keratin 5	23	Chua et al. 2000	II
krt8	Keratin 8	23	Padhi et al. 2006	II
cki	Cytokeratin type 1	19	Chua et al. 2000	I
ckl	cytokeratin type 1 like	19	Sagerström et al 2005	I
cke7	cytokeratin E7	22	Padhi et al. 2006	I

During adulthood, the same group of five keratins as described above was also found to be expressed in all layers of trunk epidermis covering the scales, as well as in the epidermis of the scale-less head. Of all six tested keratins, *cke7* was the only keratin that was expressed only at very low levels in adult epidermis (**figure 19**). These results indicated a very uniform expression of keratins in fish epidermis.

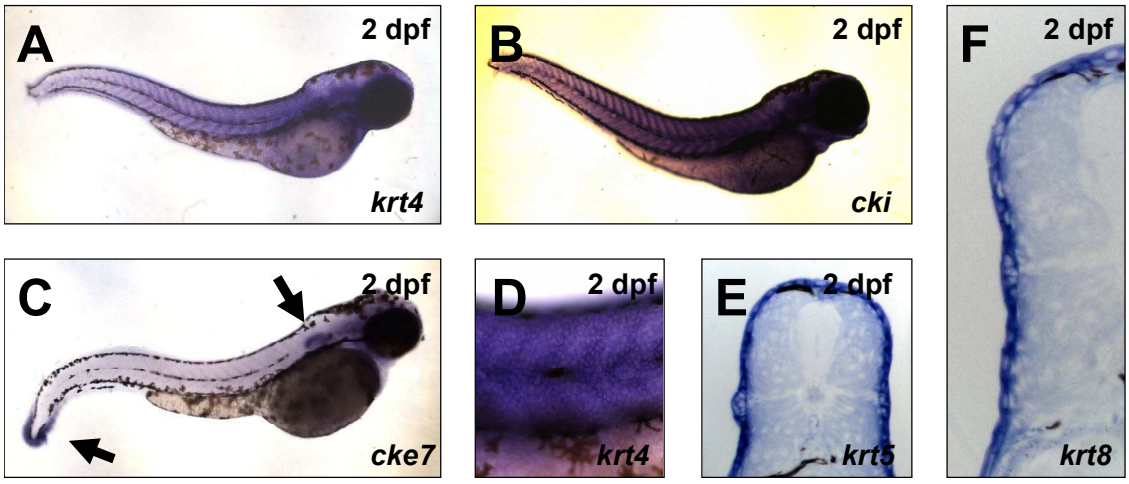
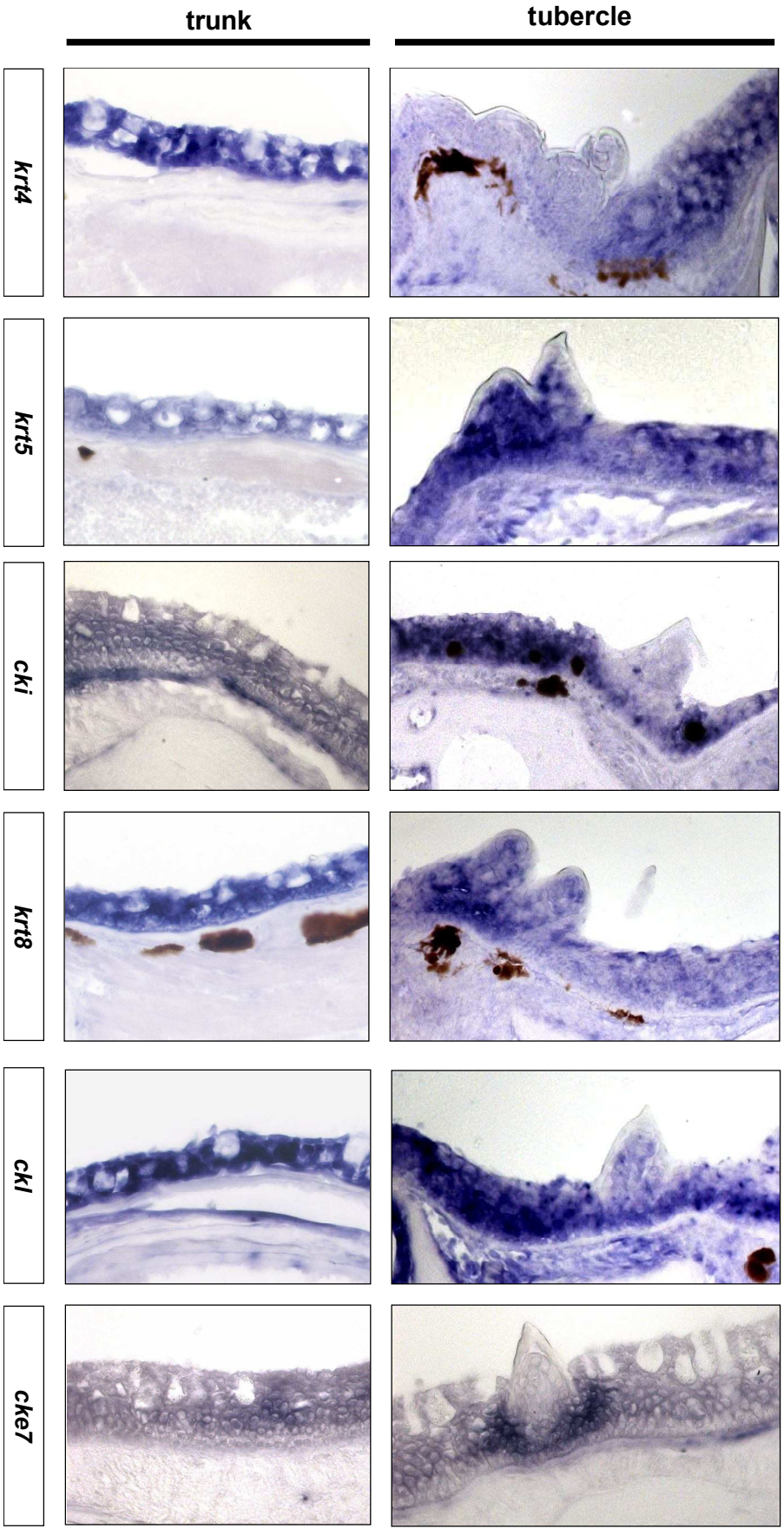


Figure 18: Expression of different keratins in larval epidermis. Six zebrafish keratins (see table 4) were tested by *in situ* hybridization on 2 dpf zebrafish larvae. Five keratins appeared to be expressed throughout both layers of the bilayered epidermis at that stage. The expression of the sixth keratin (*cke7*) was restricted to the dorsal tip of the larval fin fold and the pecfins (arrows figure C). (A-C) Lateral view of 2 dpf larva after *in situ* hybridization. (D) Magnification of the trunk region after *krt4 in situ* hybridization. (E) Cross section of a 2 dpf larva after *krt5 in situ* hybridization. (F) Magnification of the bi layered epidermis after *krt8 in situ* hybridization.

In contrast to this uniform expression in regular epidermis, keratin expression was more diverse in the area of the breeding tubercles (**figure 19**). Two of the keratins, *krt5* and *krt8,* were expressed in all layers of the tubercle including the additional upper layers. In contrast, expression of three keratins, *krt4*, *cki* and *ckl,* was reduced in the upper tubercle layers. *Cytokeratin E7* was found to be expressed

only at the base and in the epidermis around the tubercle but not (or only at very low levels) in the surrounding epidermis (**figure 19**).



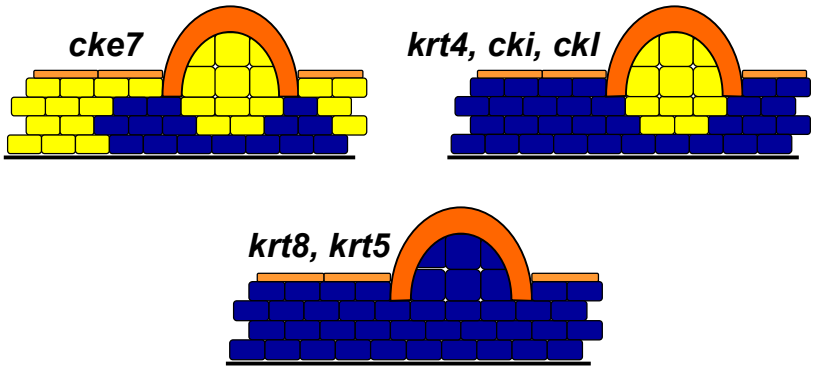


Figure 19: Expression of different keratins in zebrafish trunk and head epidermis, as well as breeding tubercles. The expression pattern in the adult epidermis of six different zebrafish keratins was assessed by *in situ* hybridization. All left panels show expression of the different keratins in the epidermis of the trunk. All keratins are expressed in all layers, except *cke7* is expressed only at weak levels. All right panels demonstrate the expression of the six keratins in regular head epidermis and in the breeding tubercels. Whereas similar as in the trunk, all keratins except *cke7* are expressed throughout all layers of the epidermis, the same keratins are differentially expressed in breeding tubercles. A summary of the different expression patterns of keratins in regular head epidermis and breeding tubercles of adults is shown at the bottom of this figure.

These results led to the conclusion that in contrast to regular zebrafish epidermis, where keratins are expressed rather uniformly, they are expressed in distinct pattern in breeding tubercles, at least similar to the mammalian keratin code. On the other side, no keratin gene was found so far that was expressed only or preferentially in the upper layers of the tubercles. According to the reaction of the pan-keratin antibody, keratin levels should be remarkably higher in these layers.

Breeding tubercle ultrastructure indicated terminal cell differentiation in breeding tubercles

Adult male pectoral fins were prepared for transmission electron microscopy (TEM) to analyse the ultrastructure and composition of breeding tubercles. Because the preparation of adult zebrafish heads turned out to be very difficult, these experiments were done only with pectoral fins. The preparation of TEM sections and the subsequent evaluation was done together with Prof. Wilhelm Bloch and Mojgan Ghilav (Deutsche Sporthochschule, Cologne).

Figures 20A-20C present semithin-sections of pectoral fin breeding tubercles. At these lower magnifications, the localisation of up to three rows of tubercles could be detected on top of the bony hemirays. The basal cells appeared rather small but more cubical and evenly arranged compared to the cells of the regular epidermis (**figure 20D**). The cells of the layers above the basal layer displayed a "star-shaped" appearance similar as in mammalian spinous layer but remained still connected to each other by numerous cell-cell contacts (**figures 20D-20F**). These spinous layer cells became progressively hypertrophic toward the outer surface. However, the cells directly underneath the cap layer (2nd tier layer) often seemed to obtain a flattened shape in parallel to the outer cap layer (**figure 20P**). The cap layer presented with very flat cells (only one cell layer thick), which were filled with a highly homogenous and electron-dense material. Cell organelles and sometimes even nuclei were only

hardly detectable (**figures 20D-20F**). In general, this continuous change in cell shape and the final flattening of the cap cells indicated the existence of a differentiation process starting in the basal layer and ending in the cap layer that is (according to the previous data) shed regularly.

Higher magnifications revealed that the basal cells are of cubic shape and arranged evenly above the basement membrane (**figure 20G**). In contrast, this cubical shape was not found in the surrounding epidermis (**figure 20D**). Hemidesmosomes could not be detected in all analysed samples. However, the basal cells were always tightly connected to the basement membrane. Additionally, basal cells were connected to neighbour cells by numerous desmosomes. Nuclei of the cells of this layer appeared healthy and not condensed (**figures 20G & 20H**).

The cells of the spinous layer (in general six to seven layers between basal layer and cap layer) increased progressively in size. Most likely due to the fixation method, the cells had undergone shrinkage while they were still connected via cell-cell contacts leading to the typical spinous appearance. Therefore, the cells between the basal layer and the cap layer will be now referred as "spinous cells". Also the nuclei of the spinous layer cells appeared healthy including cells of the tier underneath the cap layer (**figures 20E & 20F**). Spinous cells were connected to other cells including cap cells by numerous desmosomes (**figures 20I-20K**). Other cell-cell contacts like adherens junctions could not be found.

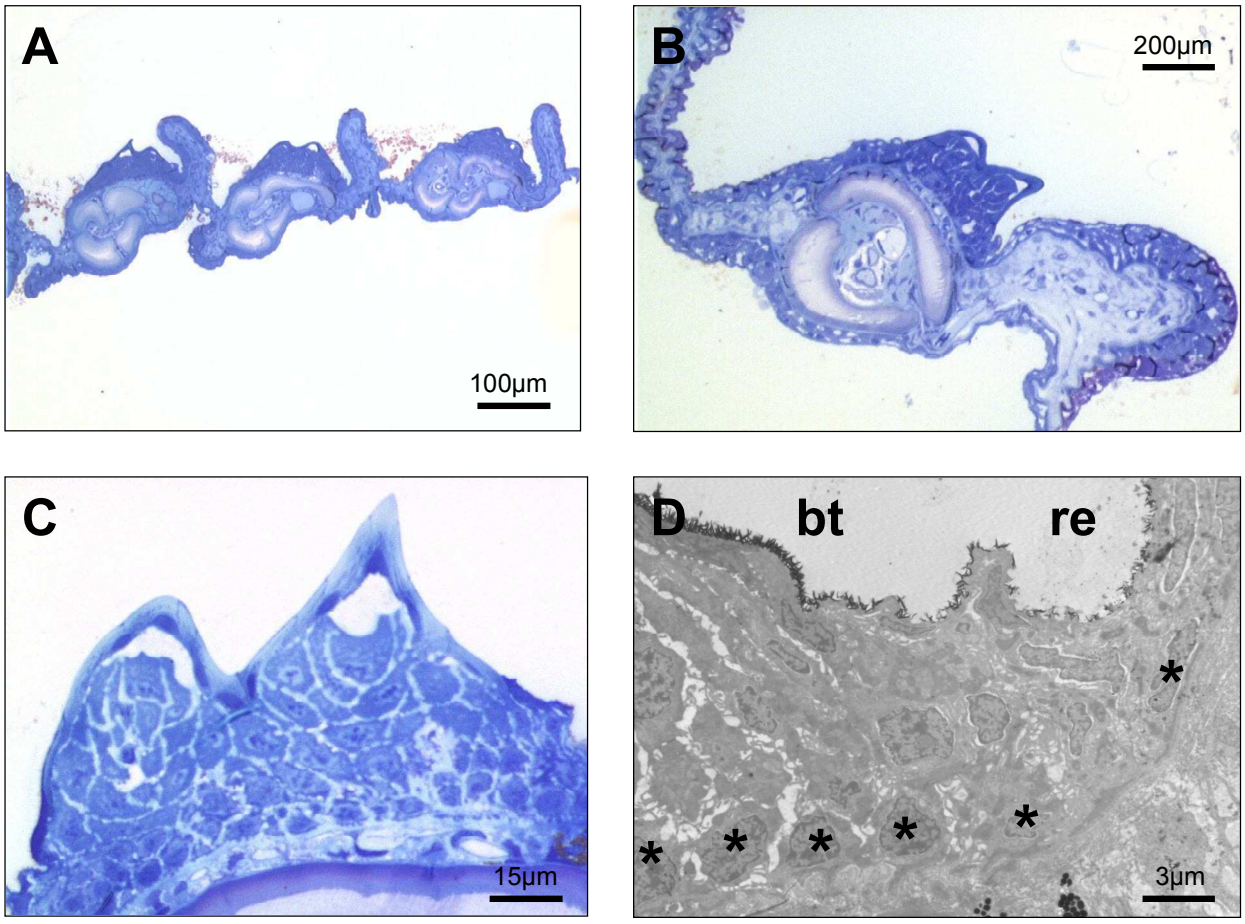
Desmosomes are normally connected to the intermediate filament system of the cytoskeleton, which are keratins in keratinocytes. However, in most of these samples keratin filaments could not be detected at all (**figure 20L**). These findings were clearly controversial to the previously described antibody staining and the keratin *in situ* hybridization data. On the other side, desmosomes often had contact with large electron-dense condensations of an unknown material (**figures 20L & 20M**). These condensations were frequently found in nearly every cell of the spinous layer but not the cap layer. Only in very few samples, filamentous structures that possibly resembled keratin filaments could be detected, especially in layers near or directly underneath the cap layer. As expected for keratin filaments, they ended in desmosomes and elongated through the cytoplasm. Probably due to the highly electron-dense material in cap cells, keratin filaments ending in desmosomes were never found in the cap layer (**figure 20N**). However, keratin filaments were suddenly orientated parallel to the outer cell surface in cap cells, possibly indicating that similar processes as the cell shape change in the *stratum corneum* of mammalian epidermis (induced by cross-linking of filaggrin and keratins) might also occur in the breeding tubercles of zebrafish (**figure 20O**). Even if filaggrin does not exist in the genome of zebrafish, other cross-linking substances might be responsible for the reorientation of keratin bundles in cap cells. Given the controversial discussion concerning keratins in fish epidermis [Henrikson & Matoltsy 1968, Burgess 1965] this might indicate that in general keratin proteins are present in epidermal cells but probably exist in a more amorphous state. However, keratin filaments were clearly detected in the epidermis of *Bagarius* [Mittal & Whitear 1979].

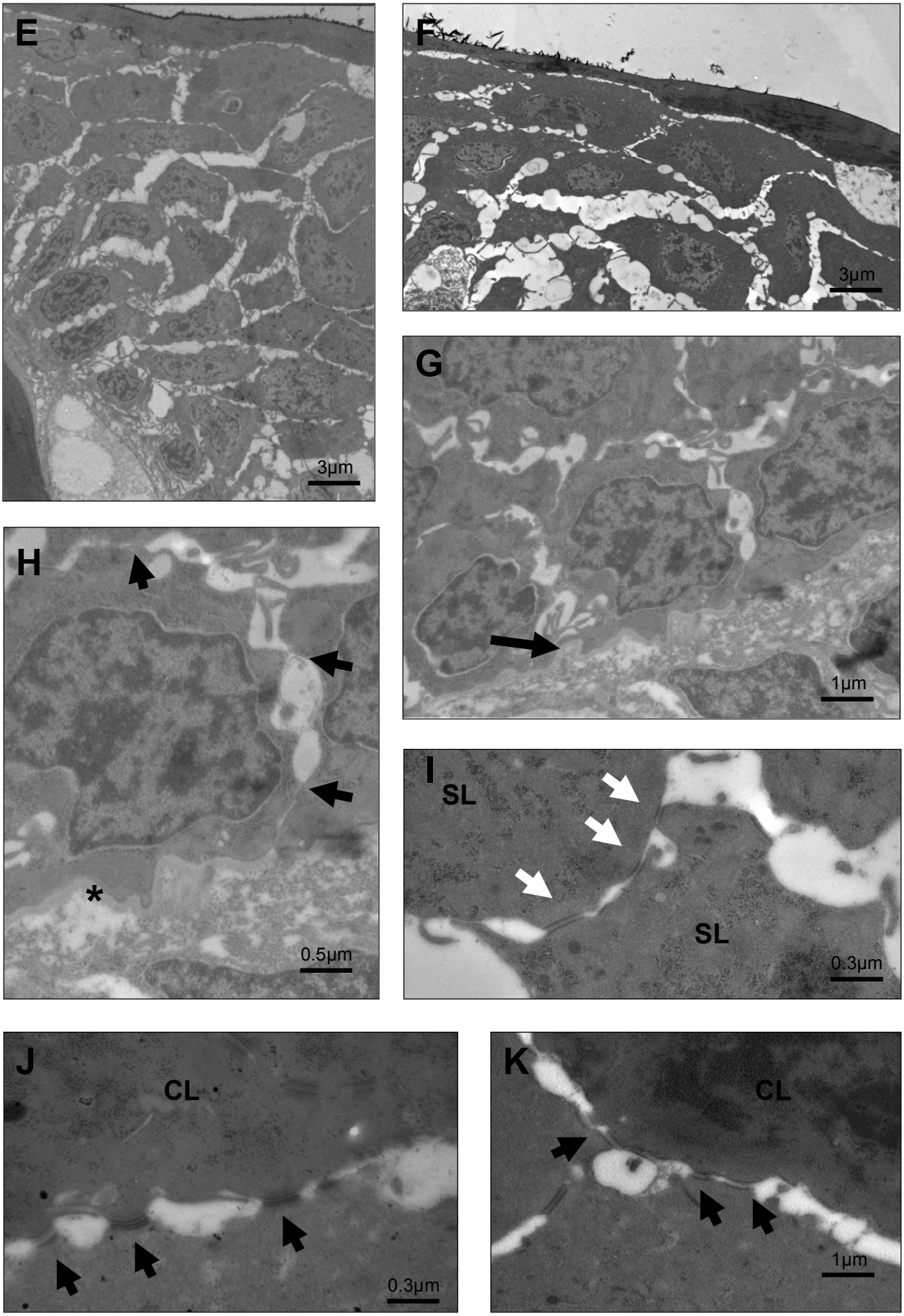
The cytoplasm of spinous layer cells was filled with increasing content of small electron-dense aggregates of unknown material and the already described large homogenous condensations (**figure**

20Q). The small aggregates were suggested to be protein condensations, perhaps ribosomes, whereas the large condensations might be non-filamentous aggregations of keratin proteins.

Starting in the cells of the granular layer, an extracellular lipid envelope is formed around cells of mammalian epidermis. Even if fatty structures are often hardly detectable, extracellular membranes, possible extracellular lipid droplets and vesicles discharging their content into the extracellular space could be observed in spinous cells in al least some of the samples (**figures 20R-20T**). These signs of lipid envelope formation were found especially between cap layer cells and the tier underneath, or between two cap cells. Interestingly, the vesicles were only found at the basal side of cap cells (**figure 20S**), in contrast to mammalian cells, where those vesicles were found only on the apical side of granular cells. These vesicles charged their unknown homogenous content into the subcorneal space, probably needed for the formation of extracellular lipid membranes or proteolytic lysis if cell-cell contacts (see below).

In regular fish epidermis, tight junctions are only found between cells of the superficial layer [Chang & Hwang 2011]. However, in the area of breeding tubercles, tight junctions were observed only between the cells of the 2nd tier, whereas the cap cells itself did have tight junctions anymore (**figures 20U & 20V**). Importantly, the same was described for the keratinized elevations of *Bagarius bagarius* [Mittal & Whitear 1979].

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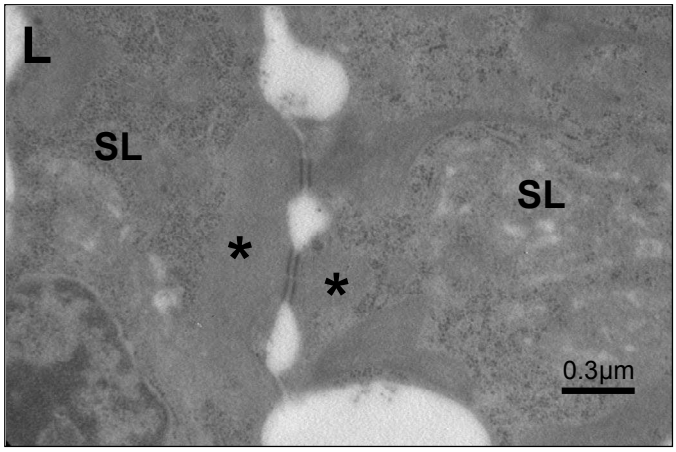
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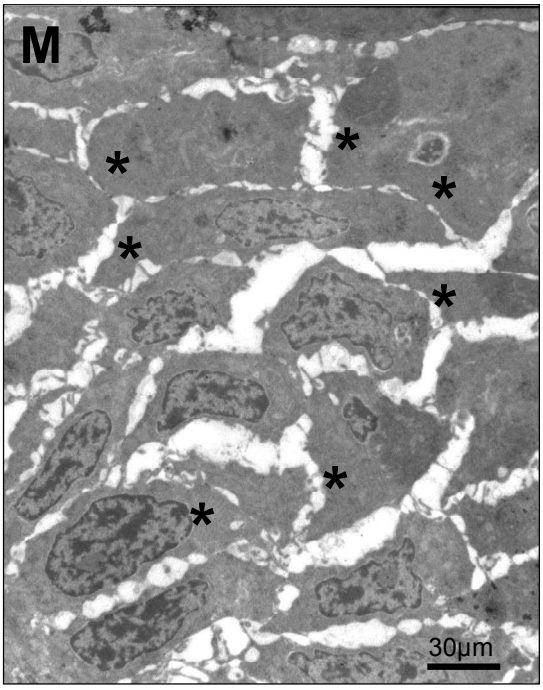
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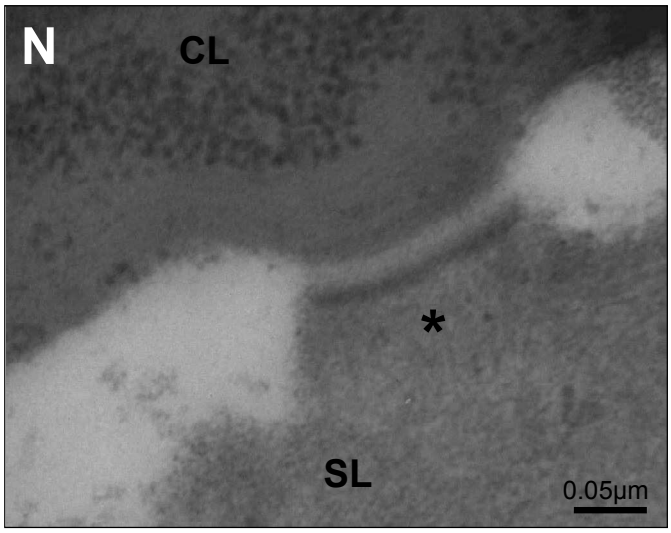
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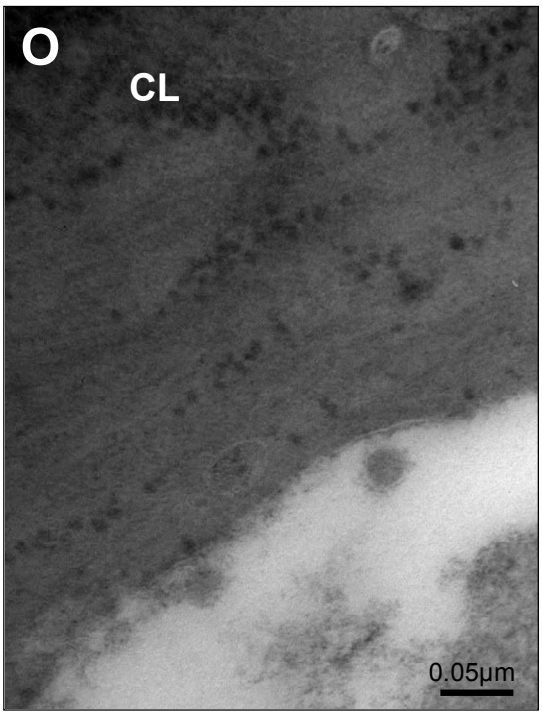
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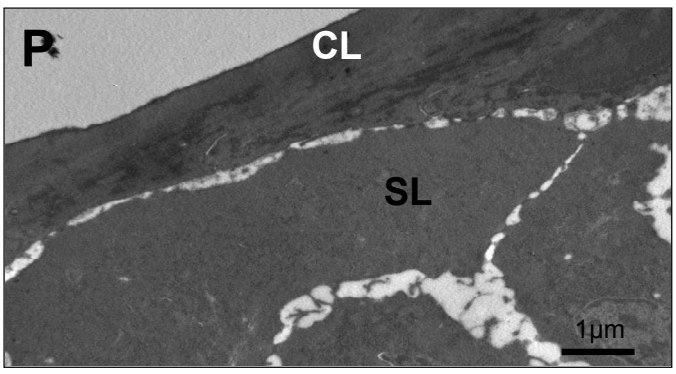
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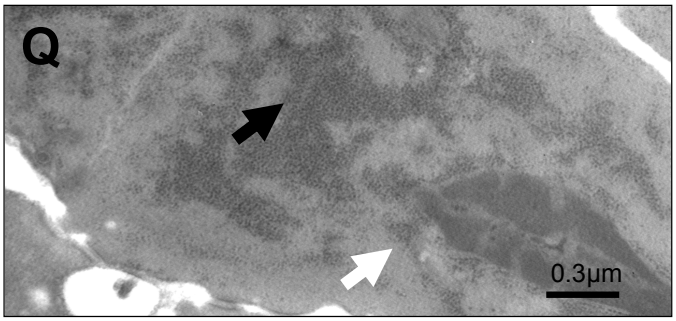
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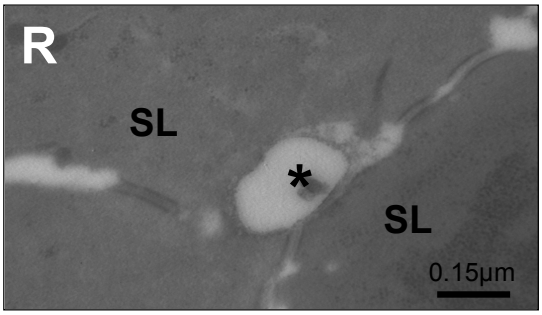
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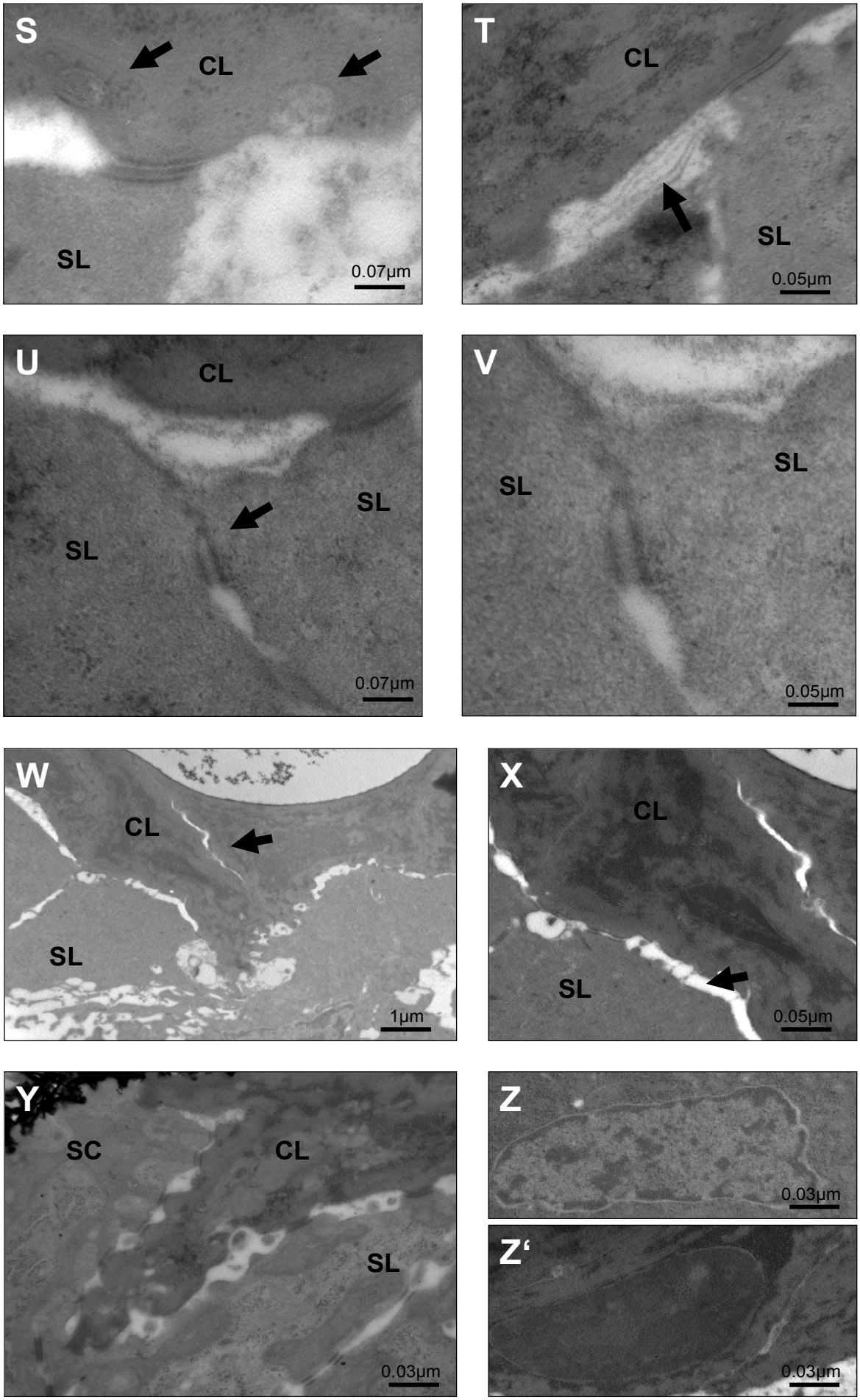
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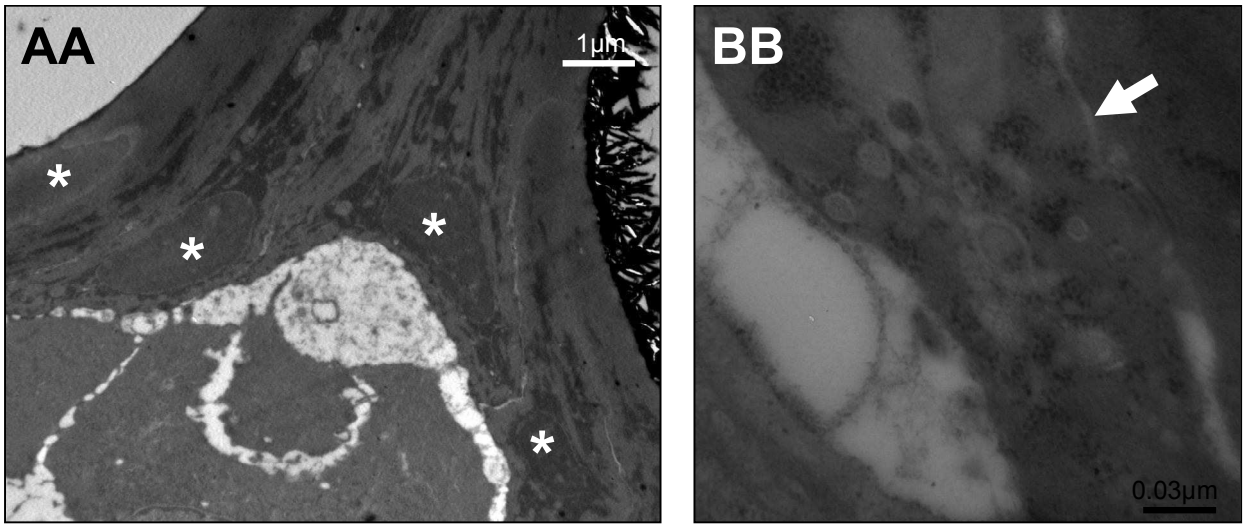

Figure 20: Differentiation of keratinocytes in pectoral fin breeding tubercles. The keratinocytes of pecfin breeding tubercles undergo a process of differentiation demonstrated by TEM imaging. (A/B) Semithin sections of tubercles at different magnifications. The tubercles are located on top of the bony hemirays. (C) Higher magnification of a semithin section. Breeding tubercles consist of regular arranged basal cells, layers of spinous cells, a 2nd tier layer and a flattened and highly electron-dense outer cap layer. (D) Interface between regular epidermis and a breeding tubercle. The more regularly arranged basal cells of the tubercle are marked by asterisks. (E) Spinous layer cells get progressively hypertrophic towards the apical surface. (F) Cap layer on top of the spinous layer consists of flat, highly electron-dense cells with often hardly detectable cell borders or organelles. (G) Basal cells of the tubercle appear more cubic as regular basal cells (basement membrane: black arrow). (H) Basal cell of a tubercle with many desmosomal cell-cell contacts to surrounding cells (black arows) and a healthy unsegmented nucleus. Basement membrane is marked by asterisk. (I) Desmosomes between spinous cells (white arrows). (J) Intact desmosomes between a cap cell and a spinous cell (black arrows) (K) Degraded desmosomes between cap and spinous cells (black arrows). The desmosomal structures might have undergone proteolytical degradation. (L) Keratin filaments running into desmosomes are only rarely seen. On the other hand, desmosomes are often in direct contact with intracellular aggregations of unknown material (marked by asterisk). (M) Homogenous condensates occur frequently in the spinous layer (marked by asterisks). (N) Only few desmosomes show keratin filaments in contact with desmosomal structures (marked by asterisk). (O) In the cap layer keratin filaments are orientated in parallel to the outer cell surface. (P) Spinous cells of the upper layers change their cell shape and flatten along the axis of the upper cap cells. (Q) Cytoplasm of spinous layer cells is filled with dots of small aggregates (black arrow) and large homogenous condensations (white arrow), both of unknown material. (R) Lipid droplets between upper spinous layer cells (marked by asterisk). (S) Vesicles at the basal side of cap cells, discharging their content into the extracellular space (black arrows). (T) Extracellular lipid layers between the cap layer and the 2nd tier cells underneath (black arrow). (U/V) In contrast to regular teleost epidermis, tight junctions (black arrow) were found in breeding tubercles only in the tier beneath the cap layer, whereas the cap itself and the layers underneath do not have tight junctions. (W/X) Cap layer furrow between two tubercles. The cell borders of the cap cells appear degraded (black arrow). A subcorneal space is present beneath the cap. (Y) Cap cell at the edge of the tubercle. The cap cell is tightly attached to the regular epidermis and the spinous cells by numerous desmosomes. (Z) Healthy nucleus of a spinous cell of the tier underneath the cap. (Z') condensed and apoptotic nucleus of a cap cell. (AA) Piling up of cap cells at the tip of the tubercle indicating high physical forces driving the formation of the tubercle. Nuclei of cap cells marked by asterisks. (BB) Summary of cap cell features compared to spinous cells: Highly electron dense and homogenous cytoplasm, vesicles at the basal side of the cell, a subcorneal space and lysed cell-cell borders. Abbreviations: **bt** breeding tubercle; **cl** cap layer; **sl** spinous layer; **sc** superficial cell; **re** regular epidermis.

Additionally, it might resemble al least partially the situation in mammalian epidermis, where tight junctions only appear in the granular layer. In this layer many important cellular changes in the process of cornification occur. Here, the cornified envelope together with the lipid envelope is formed, and cell death is initiated. The cornified layer above does not have tight junctions and is sloughed regularly [Fuchs & Raghavan 2002]. This might be summarized in the hypothesis, where 2nd tier cells have "granular-like" functions, whereas the cap layer would resemble a primitive cornified layer.

The cap cells appeared as very electron-dense and highly homogenous cell remnants, with condensed or pyknotic nuclei. The cell borders of cap cells were often hardly detectable or appeared in a degraded or lysed state (figure **20W**). However, intact desmosomes between cap cells und cells of

the spinous layer could also be detected regularly (**figure 20J**). On the other hand, the desmosomes of the cap cells seemed to be lysed with a widened extracellular space or in a state of decay in many samples (**figure 20K**), indicating proteolytic processes that might be involved in the shedding process. These degraded desmosomes might resemble the fate of corneodesmosomes in mammalian epidermis. Additionally, a subcorneal space (as often seen in amphibian) could quite often be found in those samples. This extracellular space was particularly wide in samples with degraded cell-cell contacts (**figure 20X**). On the other hand, the cap cells were tightly connected to spinous cells or superficial cells at the edge of a tubercle (**figure 20Y**).

In general, the cells of the cap layer appeared very flat compared to the hypertrophic spinous cells. In contrast to regular fish epidermis, no microridges were found in cap cells. The cytoplasm was filled with a homogenously electron-dense material. Cell organelles including mitochondria could not be found (**figure 20C, 20F, 20P & 20W**). The nuclei of cap cells always appeared condensed and showed signs of pyknosis, in contrast to the healthy nuclei of the cells underneath (**figure 20Z & 20Z'**). Overall, cap cells seemed to be metabolically inactive cells that have acquired some features of apoptotic or dying cells.

In all sections the cap layer was only one cell layer thick. However at the tip of the tubercle, several cap cells appeared in close contact (**figure 20AA**), indicating that here cap cells might be pushed on top of each other. Rupture of the tissue was often seen in the spinous layer. The flattening of cap cells on top of the tubercle might create tension to the tissue leading to the tubercle structure and the piling of cap cells seen at the tip of the tubercle. However, it would be necessary to know if such a mechanism is supported by proliferation at the base of the tubercle. Alterations of the cytoplasm membrane that might indicate the formation of a cornified envelope could not be detected. The specific features of cap cells are summarized in **figure 20BB**.

In summary, the results of the ultrastructural analysis of pectoral breeding tubercles indicate a differentiation process in tubercles that leads to the formation of the cap layer. The most dramatic steps occur in the 2^{nd} tier layer (that might have similarities to the mammalian granular layer), including cell shape changes and the formation of tight junctions. The outermost layer consists of inactive and probably dying cells that still have remained their nuclei in contrast to mammalian corneocytes. The formation of a lipid envelop is another strong parallel to cornification. However, a cornified envelope was not detected.

In contrast to breeding tubercles, the surrounding epidermis appeared rather thin at pectoral fins, with only three to four cell layers in total (**figure 21**). In general, the tissue appeared more compact compared to tubercles. Additionally, the star-shaped form of cells of the spinous layer could not be found in the surrounding epidermis.

As mentioned before, the basal cells of the surrounding epidermis displayed a flatter appearance compared to the base of the tubercles and were more irregularly arranged (**figures 21A & 21B**). Mucous cells only occurred in the regular epidermis, but never in the area of breeding tubercles. As mucous cells are typical for mucogenic epithelia but do not occur in keratogenic epithelia, this again

points towards the model of a "keratogenic" island (the tubercle region) surrounded by regular mucogenic epidermis (**figure 21C**).

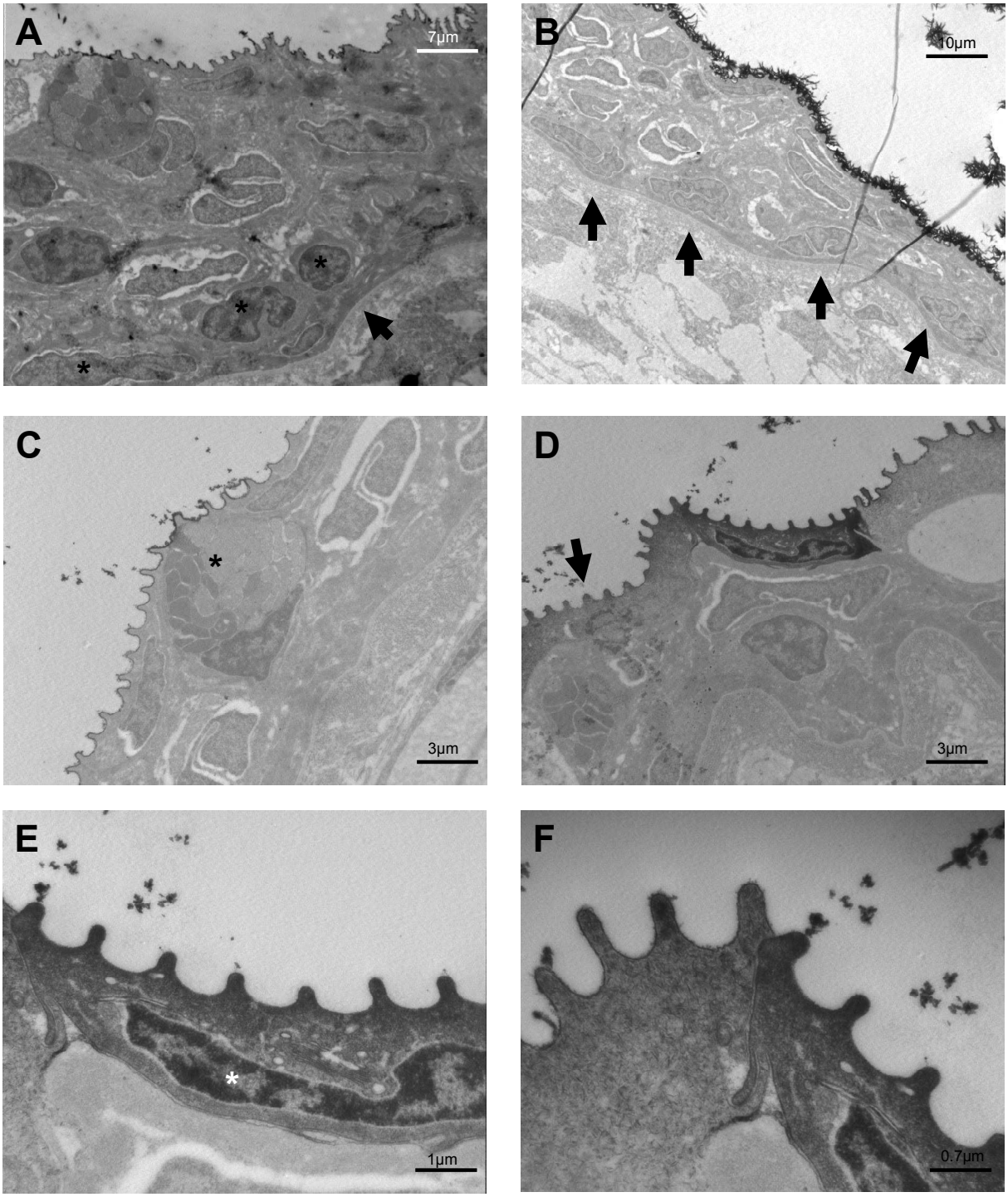

Figure 21: Ultrasturcture of regular epidermis of adult zebrafish pectoral fins. (A/B) Overview of regular epidermis of the pectoral fins. In contrast to breeding tubercles, the epidermis is thinner: four to five tiers (figure A) and three to four (figure B) respectively. The flat and segmented nuclei of basal cells are highlighted by asterisks in figure A. The basement membrane is marked in figure B by black arrows. (C) Mucous cells (asterisk) are only found in regular epidermis but not in the area of breeding tubercles. The outer surface of superficial cells displays the typical microridge pattern. (D) The cells of the superficial layer are flat but healthy cells with an intact nucleus. Microridges are always present (black arrow). (E) Nucleus of a superficial cell. (F) Superficial cells are tightly connected to each other by numerous tight cell-cell contacts.

The superficial cells appeared flat, but in contrast to cap cells presented with a healthy, segmented nucleus and intact cell organelles (**figures 21D & 21E**). The cells in this layer were tightly attached to each other and the cells of the intermediate layer. A subcorneal space was not found. The apical side of superficial cells showed the characteristic microridge structure (**figures 21D-21F**), which was completely absent in cap cells.

Despite the existence of numerous tight cell-cell contacts between cells of the superficial layer (**figure 21F**), a definite proof of tight junctions could not be made by electron microscopy. Therefore, immunohistochemistry was performed with a mouse anti-ZO1 antibody. The antibody was directed against *zonula occludens* protein 1, a protein of the tight junction complex. Staining of regular epidermis of *krt4::GFP* transgenic fish revealed that the cell outlines stained via the antibody correlated with the GFP positive superficial cells (**figure 22A & 22B**). However, the antibody did not recognize tight junctions in the breeding tubercles, which were probably shielded by the cap layer.

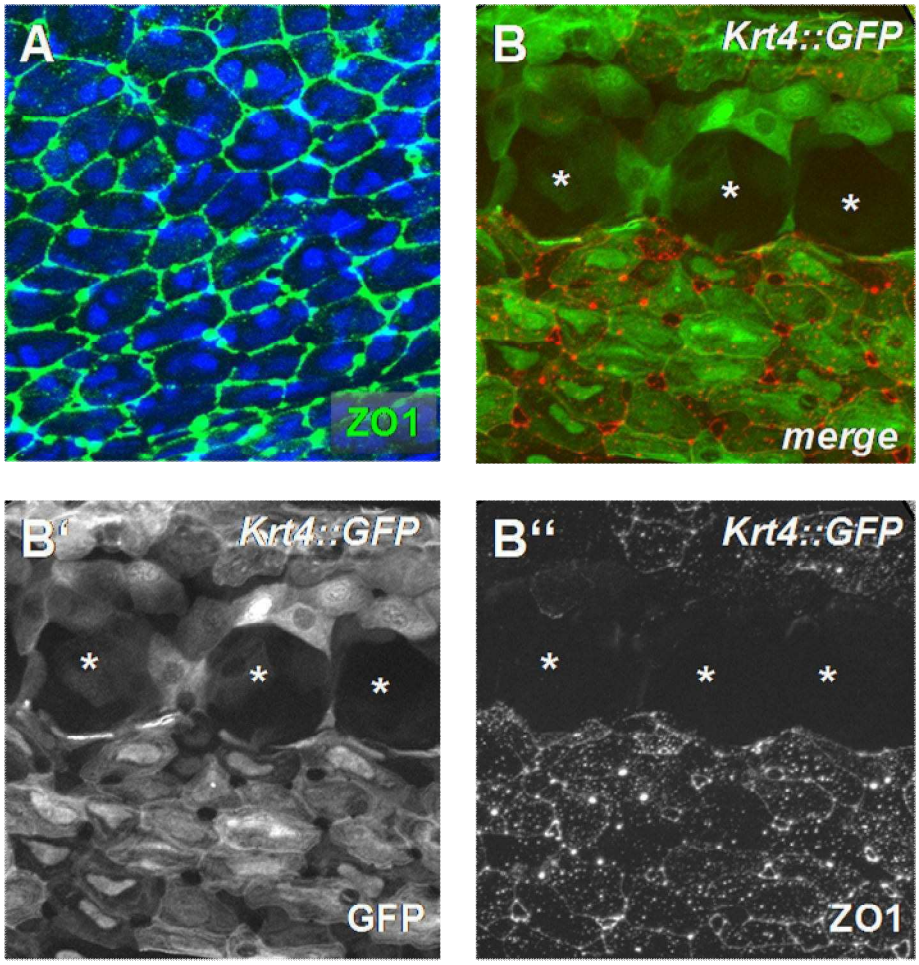

Figure 22: Tight junctions in zebrafish epidermis. Tight junctions in the epidermis of zebrafish whole-mount pectoral fins were visualized by anti-ZO1 antibody. (A) Regular epidermis of the fin. Tight junctions seal the cells of only one cell layer. (B) Tight junctions are found between cells of the superficial layer in regular epidermis as demostrated by immunostaining in *krt4::gfp* transgenic fish. However, the antibody did not detect tight junctions in the 2nd tier of breeding tubercels, where GFP expression is lost (marked by white asterisks). B merge, B' GFP channel, B'' Cy3 channel shows ZO1 antibody staining.

In summary, the TEM data and the ZO1 immunostaining indicated crucial differences between the cells of the breeding tubercle compared to the cells of regular epidermis. In tubercles, tight junctions occur in the course of a ("cornification-like") differentiation process similar as in mammalian epidermis, whereas in regular epidermis the location is different, but as the EVL gets replaced by basally derived cells, the occurrence of tight junctions in the superficial layer might be part of the second (regular) differentiation process in fish epidermis.

Proliferation is increased in breeding tubercles but also restricted to the *stratum germinativum*

We wondered if the processes leading to tubercle formation include not only differentiation but also effect proliferation in comparison to the surrounding epidermis. We speculated that proliferation might be increased because of the additional layers of tubercles, or even decreased because cells rather differentiate than proliferate. To address this question we performed BrdU incorporation assays over the time period of 24 hours. Incorporated BrdU was visualized by immunostaining and the percentage of BrdU positive nuclei in the breeding tubercle regions was compared to BrdU incorporation in regular epidermis.

The age of the tested fish was 60 dpf and general tubercle formation should already be completed at that age. However, it could clearly be seen that proliferation was increased in the area of breeding tubercles (**figure 22A**). This result was additionally confirmed by estimation of the ratio: BrdU-positive nuclei / all nuclei in the region of the tubercles compared to the ratio in regular epidermis (**figure 22B**).

In regular epidermis, BrdU positive cells were found throughout all layers, confirming that proliferation occured throughout the whole intermediate layer. However, this pattern was not found in breeding tubercles. Even if the proliferation rate was higher in breeding tubercles, the BrdU positive cells were concentrated in the lower layers of the epidermis, indicating that in tubercles proliferation is more restricted to the *stratum germinativum*. This result argues for a similar architecture of breeding tubercles and mammalian epidermis, together with the TEM data and the shedding process. Additionally, it clearly highlights the difference between regular epidermis in fish and breeding tubercles (**figure 22A**). In accordance with the TEM data, cells in the tubercles proliferate only in the basal or lower spinous layer. In the following they exit cell cycle and start a differentiation program.

This result could also be confirmed by antibody staining with the mouse (clone 4A4) anti-p63 antibody. However, this antibody did not discriminate between the different isoforms of p63, but because the ΔN isoforms should be the much more abundant than the TA isoforms, we argued that the antibody would stain preferentially nuclei of cells that still have the capacity to proliferate. **Figure 23** demonstrates that in regular epidermis p63 positive nuclei could be found throughout the whole epidermis, in accordance with BrdU incorporation.

Furthermore, p63-positive cells were restricted to the lower layers in breeding tubercles. This strongly indicates a more stratified architecture in breeding tubercles as in regular fish epidermis. Additionally it points to the existence of a differentiation process in tubercles. Descriptions of breeding tubercles in other fish species also mention this different pattern of proliferating cells [Mittal & Whitear 1979].

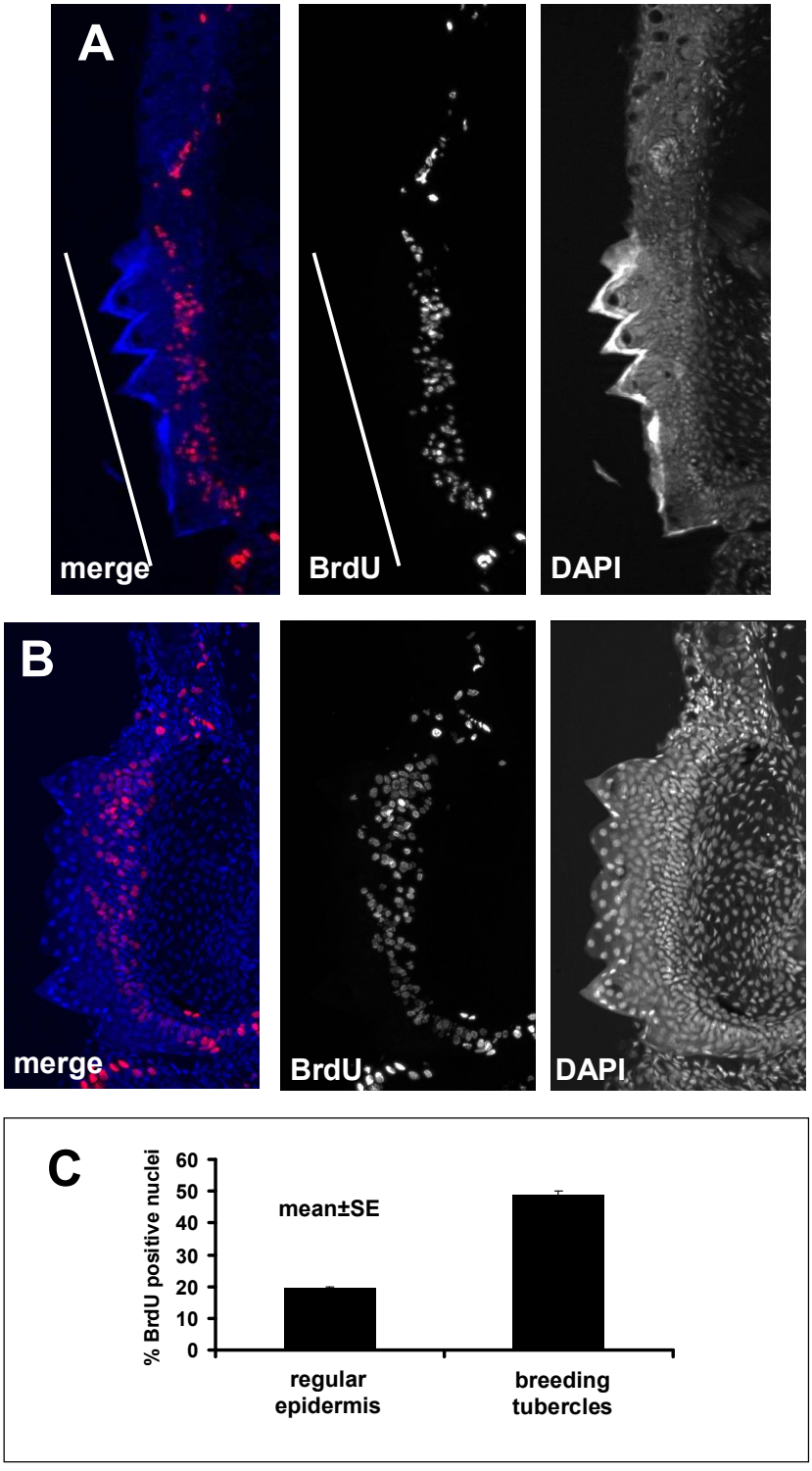

Figure 22: Proliferation is increased at the base of breeding tubercels compared to regular surrounding epidermis. BrdU incorporation assay was perfomed to compare proliferation in regular epidermis to breeding tubercels. (A/B) Two examples of breeding tubercle regions are shown together with the surrounding regular epidermis. The BrdU channel demonstrates the increased proliferation in the tubercle area (area marked by white bars in A). In regular epidermis, proliferation occurs in all layers of the intermediate layer, whereas in breeding tubercels proliferation is reduced to the *stratum germinativum*. (C) Quantification of the proliferation rate in breeding tubercles and the regular epidermis. Graph shows the ratio BrdU-positive nuclei/all nuclei (counted via DAPI) in percent.

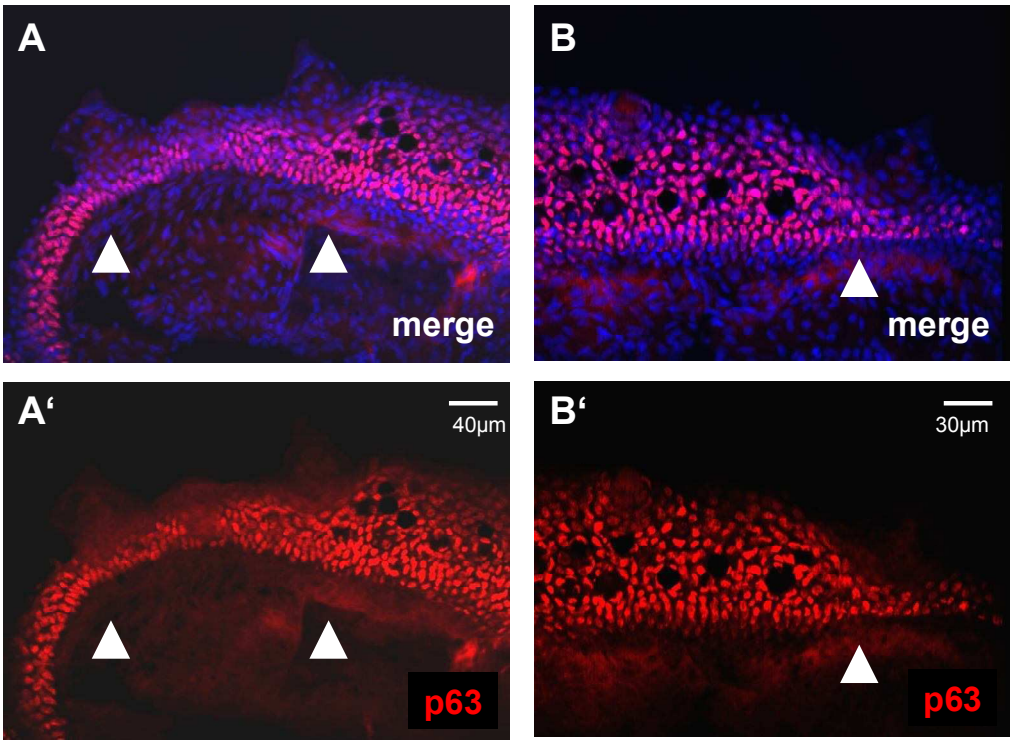

Figure 23: At the base of breeding tubercles, proliferation is found only in the basal layer in contrast to regular epidermis. Immunostaining with the anti pan-p63 antibody revealed that in regular epidermis, cells troughout the whole intermediate layer have remained the capacity for proliferation, whereas the p63-positive domain was found to be reduced to the basal and the lowest spinous layer cells in breeding tubercels.. (A/B) merge; (A‘/B‘) Cy3 channel. White arrowheads mark the tubercle domain.

Non-apoptotic functions of Caspase 3 in the outer layers of breeding tubercles

Cornification in mammals and amphibians involves a specific form of cell death, but still shares similarities with apoptosis. As the nuclei of cap layer cells appeared condensed and inactive, and cell organelles, in particular mitochondria were not found in TEM images of cap cells, we wondered if a similar process of cell death takes place in fish epidermis that is possibly more similar to apoptosis.

Acridine orange *in vivo* staining of adult zebrafish, as a general indicator of cell death, failed to detect cell death in breeding tubercles (**figure 24A**). The very weak fluorescence of the cap of tubercles was considered an auto-fluorescence artefact, as it was detected with all fluorescence filters (data not shown).

Next we analysed the epidermis of whole-mount male pectoral fins via TUNEL assay and sectioned them after plastic embedding.

In general, TUNEL-positive nuclei were only scarcely detected in all samples indicating very low levels of apoptosis in adult fish epidermis. Importantly, no TUNEL-positive nuclei were found in the cap layer of breeding tubercles arguing against elevated apoptosis in the cap layer (**figure 24B & 24C**). Few apoptotic nuclei in breeding tubercles were found in basal cells or in the mesenchyme below the tubercles. However, this result demonstrated only that no DNA single strand breaks occurred in cap cells of the breeding tubercles. A different type of cell death might still occur or the cells of the cap

layer just get metabolically inactive while their nuclei remain largely intact. Additionally, even mammalian *stratum corneum* cells are not TUNEL positive [Gandarillas et al. 1999].

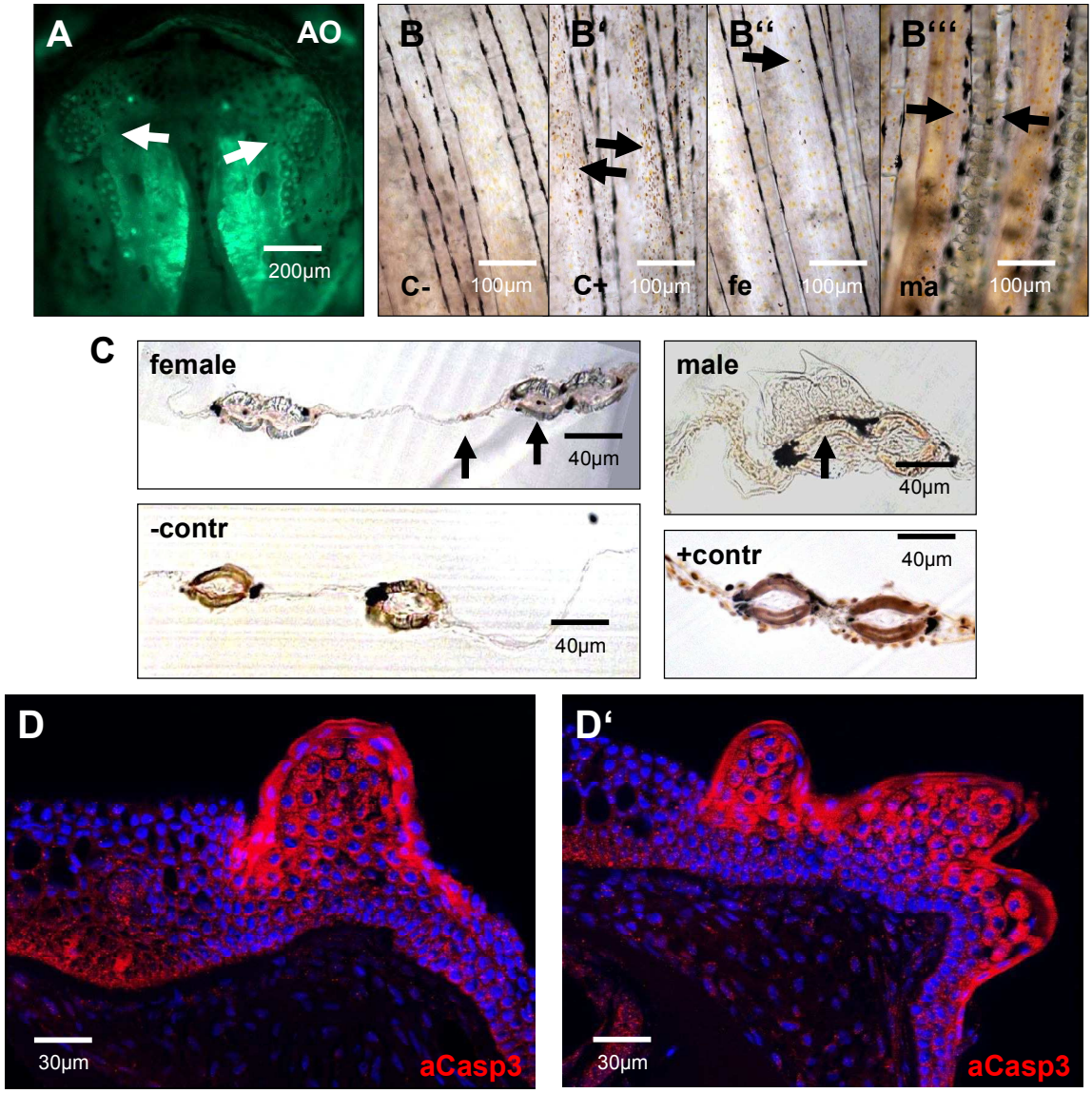

Figure 24: Cap cells of breeding tubercles do not show a positive reaction in cell death assays, but display high levels of active Caspase 3. (A) Acridine orange (AO) staining does not inidicate cell death in the tips of breeding tubercles. White arrows point to tubercle plates. (B) TUNEL assay of whole-mount pectoral fins reveales that cap cells of breeding tubercles are negative for TUNEL reaction. Black arrows point to TUNEL positive nuclei. ma: male; fe: female; C-: negative control; C+: positive control. (C) TUNEL stained pectoral fins were plastic embedded and sectioned. Again no TUNEL positive nuclei are found in the cap layer. (D/D') Immunostaining of breeding tubercles and the surrounding epidermis using an active Caspase 3 antibody. Caspase 3 is found to be highly active in the upper spinous layers, the 2nd tier layer and the cap layer.

To further investigate this point, we exploited a mouse antibody directed against active Caspase 3. Caspase 3 is one of the major effector caspases in the classical apoptosis cascade, where it is activated by other upstream caspases [Snighda et al. 2012, Taylor et al. 2008]. Immunohistochemistry was performed on paraffin sections of adult zebrafish heads and pecfins.

Caspase 3 was found to be highly active in breeding tubercles. However, Caspase 3 was activated not only in cells of the cap layer (as expected according to the TEM results) but also in the upper spinous layers and the 2nd tier layer, which showed no signs of apoptosis in TEM and in the previous histological methods (**figures 24D & 24D'**). Additionally, the intensive staining of the cap layer contradicts to the results of the TUNEL assay.

However, a lot of recent publications claim different functions of caspases apart from their most commonly know function in apoptosis, including differentiation of keratinocytes in mammalian epidermis [Wu et al. 2011, Lamkanfi et al. 2007, Okuyama 2004, Schwerk & Schulze-Osthoff 2003]. The strong presence of cleaved Caspase 3 together with the healthy appearance of spinous layer cells in the TEM images might lead to the suggestion that Caspase 3 has non-apoptotic functions in zebrafish breeding tubercles. Due to the fact that the pattern of activated Caspase 3 staining widely overlapped with the layers of differentiating cells in the tubercles, one might conclude an important function of Caspase 3 in the differentiation process of epidermal cells in breeding tubercles.

Cap cell membranes display leakiness in barrier assays pointing towards cell degradation

Regular epidermis is generally impermeable to substances from the outside. In mammals, this is mainly achieved by the *stratum corneum*, but also the tight junctions of the granular layer contribute to this function. In our lab we use methylene blue to study re-epithelialization after wounding of adult zebrafish. Generally, methylene blue is not able to pass the epidermis [Richardson et al. 2013]. To test whether the tight junctions in the tier below the cap layer efficiently seal the epidermis we used this barrier assay to stain zebrafish breeding tubercles.

Interestingly, tubercles could easily be stained by methylene blue presumably due to the detached cap layer (**figures 25A &25B**). However, the staining was washed away again very quickly, indicating that the dye might only reach the subcorneal space. The staining performed very well on the bigger jaw tubercles, whereas it was only very weak for the pecfin tubercles.

However, it turned out that tubercles could be stained much more efficiently by another vital dye, calcein, which is normally used to target bone tissue [Du et al. 2001]. Wildtype fish were stained with calcein alive and afterwards anaesthetized and used for whole-mount imaging or sacrificed and prepared for cryo-sectioning. In contrast to methylene blue, calcein stained the tubercles much stronger and with long lasting effect (**figures 25C-25E**). Sections revealed that the layers underneath the cap layer were indeed tightly sealed. The dye had not entered the tiers underneath the cap layer. However, the calcein signal was very strong in the cytoplasm of the cap cells themselves (**figure 25F-25H**). As we considered the cap layer cells as metabolically inactive or perhaps even dying, we concluded that the cell membranes might be leaky, thereby allowing to dye to pass the membrane barrier. Degradation of cell membranes of cap cells was already confirmed by TEM imaging.

Again, this would argue against an apoptotic mechanism in cap cells, as cell membranes remain intact during apoptosis [Lippens et al. 2005].

In the following, we used the calcein stain to study cap layer regeneration during a shedding cycle and tubercle formation in mutants.

According to recent data from Manuel Metzger in our laboratory, shedding of the cap layer occurs quite frequently in most tested fish. However, the time until a completely regenerated cap layer formed was very variable in different individuals. The regeneration period ranged from five days up to two weeks. The underlying reasons for this variability still remain unknown.

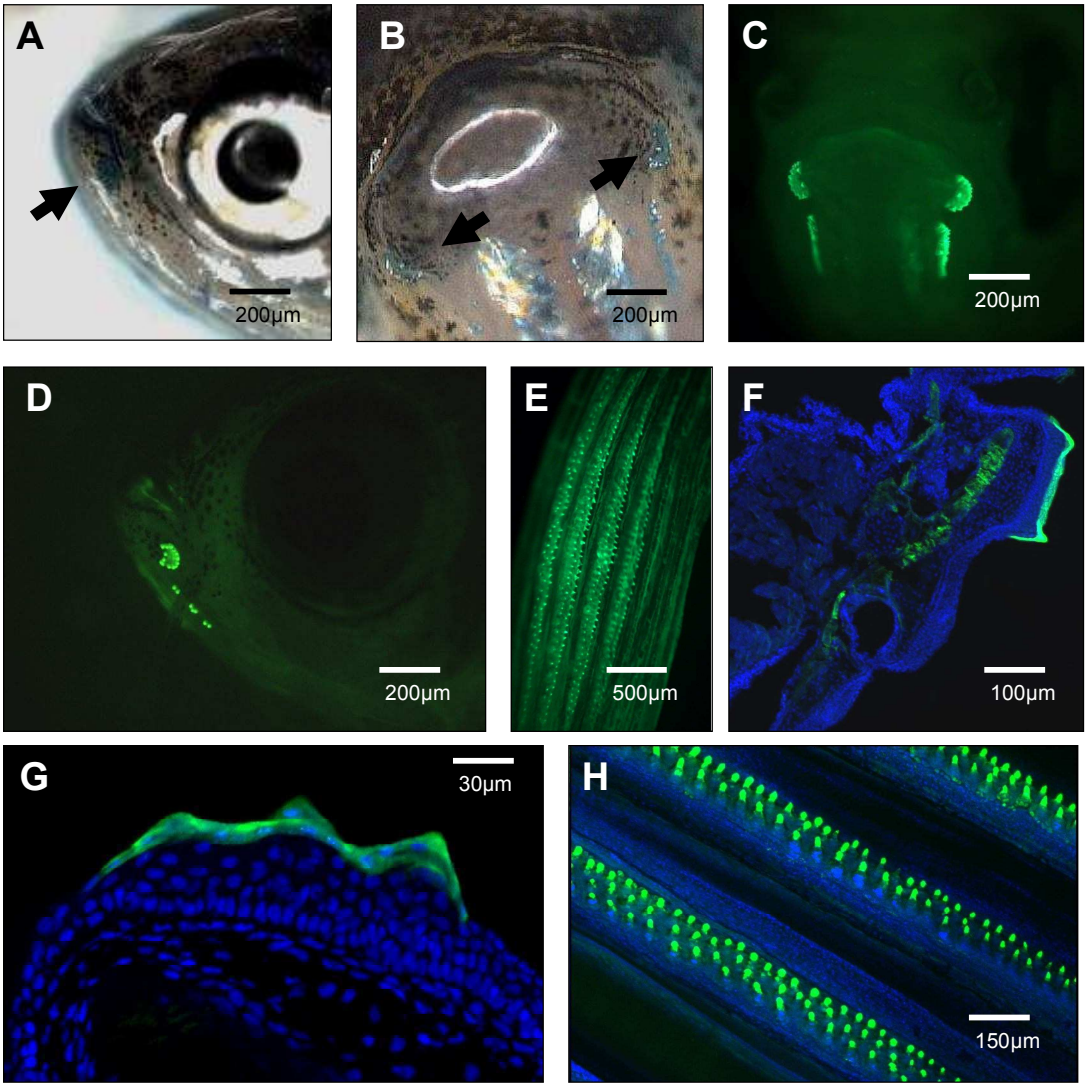

Figure 25: Cells of the cap layer can be stained with vital dyes like methylene blue or calcein. (A) Lateral view of a zebrafish head. Methylene blue stains the tubercle plate (arrow). (B) Ventral view of the head. Tubercle plates on both sides are stained by methylene blue. (C/D) Lateral and frontal view of the head of an adult zebrafish stained with calcein. The tubercles are brightly stained by this dye. (E) Additionally the tubercles on the pectoral fins are stained with calcein. (F/G) Sections of calcein stained fish reveal that calcein enters the cytoplasm of the cap cells but not the tissue underneath the cap layer. (H) Calcein stained cap cells of pectoral fin tubercles.

Transglutaminase 1 is specifically expressed in the upper layers breeding tubercles

Transglutaminases (TGM) are crucial for cross-linking keratins and CE associated proteins in mammalian cornification resulting in the formation of a dense keratin network and the cornified envelope [Fuchs & Raghavan 2002]. Only very few data are available concerning transglutaminases in

zebrafish [Deasey et al. 2012]. Additionally, these data revere only to embryonic/larval development, whereas the functions in adult fish are not analysed so far.

tgm1 knock-out mice die around birth due to *stratum corneum* defects [Matsuki et al. 1998]. As transglutaminases are crucial for cornification in mammals, we wanted to find out if they are also involved in the differentiation process of tubercle keratinocytes. To address this suggestion, we used an *in situ* RNA antisense probe to detect expression of *tgm1* in the epidermis of adult zebrafish.

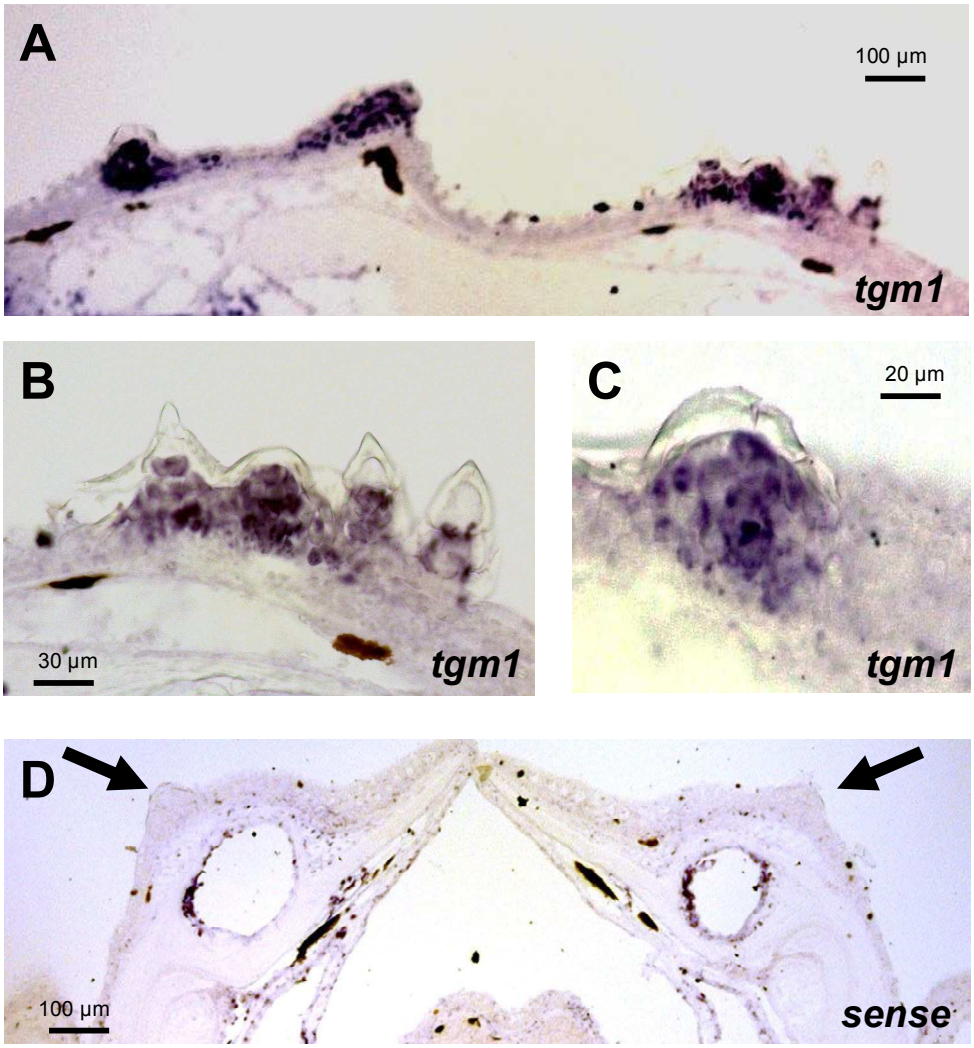

Figure 26: Expression of *transglutaminase1 (tgm1)* in breeding tubercles. (A-C) Expression of *tgm1* (*in situ* hybridization data using the *tgm1* RNA antisense probe) is found exclusively in the upper cells of breeding tubercles, whereas regular epidermis and lower layers of tubercle remain unstained. A – overview of staine d tubercles of the lower jaw. B – staining of larger tubercles of the head. C – staining of small (probably newly developing) tubercle. (D) No staining was obtained with the *tgm1* sense probe. Not stained tubercles are marked by black arrows.

tgm1 was found to be completely absent in regular epidermis of adult zebrafish, but was highly expressed exclusively in the upper layers of breeding tubercles (**figures 26A-26C**). This would indicate that *tgm1* is expressed only in those cells that differentiate in tubercles, whereas the proliferating cells at the base of tubercles and in regular epidermis are *tgm1* negative. The staining was not found with the corresponding sense probe (**figure 26D**). Even if the functions of

Transglutaminase 1 were not investigated further, the highly restricted expression pattern strongly suggested participation of TGM1 in the differentiation of epidermal cells in zebrafish breeding tubercles. Together with active Caspase 3, *tgm1* is therefore a specific marker for differentiating keratinocytes in the breeding tubercles of zebrafish.

Differentiation of zebrafish keratinocytes in breeding tubercles strongly depends on Notch signalling and activation of Caspase 3

Notch signalling is especially known for the process of lateral specification and is frequently involved in processes, where a single cell is chosen to adopt a different fate than the surrounding cells [Panelos & Massi 2009, Okuyama et al. 2008]. As already described, Notch signalling plays important roles during keratinocyte differentiation in mammals.

The Notch signalling pathway is activated through binding of membrane-bound Notch ligands of surrounding cells to the Notch receptor. In vertebrates, two types of Notch ligands are known: Delta-like and Jagged. Especially Jagged is known to be expressed in mammalian epidermis. Jagged 2 was detected in basal cells, whereas Jagged 1 seemed to be expressed only in suprabasal cells. After epidermal cells exit from the cell cycle, they initiate expression of Notch. Binding of Jagged 1 ligand to the Notch receptor leads to cleavage of the NICD (Notch intracellular domain) by γ-secretase, which is then released into the nucleus. There, a complex of NICD and RBP-J binds to responsive target promoters and drives the expression of Notch target genes which are then responsible for the specific Notch functions [Okuyama et al. 2008, Watt et al. 2008, Nickoloff et al. 2002].

To study a putative function of Notch signalling in the formation of breeding tubercles in zebrafish, we took advantage of the chemical inhibitor DAPT, which is a specific γ-secretase inhibitor and frequently used in zebrafish and other model-organism or cell culture [Okuyama et al. 2004, Geling et al. 2002]. As Notch signalling was already linked to a non-apoptotic function of Caspase 3 during keratinocyte differentiation and cornification [Mack et al. 2005, Okuyama et al. 2004], we also used z-DEVD, a Caspase 3 specific inhibitor [Valencia et al. 2008, Parng et al. 2004].

Approximately 15 to 20 juvenile zebrafish were kept in 100 µM DAPT, 10 µM z-DEVD or 0.2% DMSO as control for three weeks during the development of breeding tubercles. The experiment was started when the fish had reached an age of 20 days and ended at 41 dpf. According to previous data it was assumed that tubercles had not been developed at the start, but should definitely exist at the end of the experiment. Afterwards tubercle formation was evaluated by calcein staining or histological HE staining. Measurement of standard length after the treatment ensured the same developmental progress in treated and control groups.

Both inhibitors were found to reduce or even completely block tubercle formation (**figure 27A**). The experiment was repeated three times in the above described way. In the overall evaluation, 65% of the fish in the DMSO control group had clearly developed jaw breeding tubercles of an age-respective size. The remaining 35% had developed tubercles as well, but remarkably smaller and/or reduced in

number. In all experiments, no juveniles without tubercles were detected in the control groups. Inhibition of NICD cleavage by the γ-secretase inhibitor DAPT reduced the amount of fish with normal-sized tubercles to 44%. Additionally, 29% displayed only poorly developed tubercles and 27% even had no tubercles at all. Caspase 3 inhibition yielded in 28% of fish with normally developed tubercles, 51% with small or less tubercles and 21% without any tubercles. The data of this analysis and examples for regular-sized and small-sized tubercle structures are shown in **table 5** and **figure 27B**.

Table 5: Chemical inhibitor treatment reduces breeding tubercle formation.

Treatment for three weeks						
Fish with:	**DMSO**		**DAPT**		**z-DEVD**	
no BT	0	0 %	9	27 %	8	21 %
small BT	11	35 %	10	29 %	20	51 %
regular BT	20	65 %	15	44 %	11	28 %
Total	21	100 %	34	100 %	39	100 %
Treatment for four weeks						
Fish with:	**DMSO**		**DAPT**		**z-DEVD**	
no BT	0	0 %	2	11 %	2	10 %
small BT	7	37 %	12	63 %	14	70 %
regular BT	12	63 %	5	26 %	4	20 %
Total	19	100 %	19	100 %	20	100 %

Treatment over three weeks was done three times in independent experiments. Treatment over four weeks was done only once. The first value of each section is the total number of juveniles; the second number the relative value in percent.

To demonstrate that the above described results are not only caused by delayed development due to a toxic effect of the inhibitor solutions, the standard length of the juveniles was measured after the experiment to demonstrate comparable developmental progress in all groups according to the standard table published by Parichy et al. in 2009 (**figure 27D**). Additionally, the same experiment with both inhibitors was performed over four weeks instead of three, again to exclude a general developmental delay. Again, the effect of both inhibitors could be confirmed (**figures 27C & figure 27E**). Therefore, the described effect of the two inhibitors should be due to the inhibition of Notch signalling or Caspase 3 respectively and not due to a general developmental delay.

For closer evaluation, inhibitor treated juveniles were analysed by hematoxylin/eosin staining and active Caspase 3 immunostaining. HE staining was performed to demonstrate the absence or reduction of tubercle development in treated fish, and additionally to compare the structure of tubercles in inhibitor treated fish to control fish. Special focus was put to the question if a cap layer was still able to form after blockage of active Caspase 3 or Notch signalling in general. Additionally we speculated that, as zDEVD inhibits Caspase 3 activation, active Caspase 3 should not be detectable anymore in the zDEVD treated group. However, if Caspase 3 is activated by Notch signalling, activation of Caspase 3 should be blocked in the DAPT treated group as well.

Figure 28A demonstrates that tubercle size was decreased or completely absent in DAPT or zDEVD treated fish in comparison to the DMSO control group. However, both groups presented with a fully developed cap layer on top of the (smaller) breeding tubercles. This would indicate that Notch signalling and Caspase 3 are important but not absolutely crucial for breeding tubercles formation.

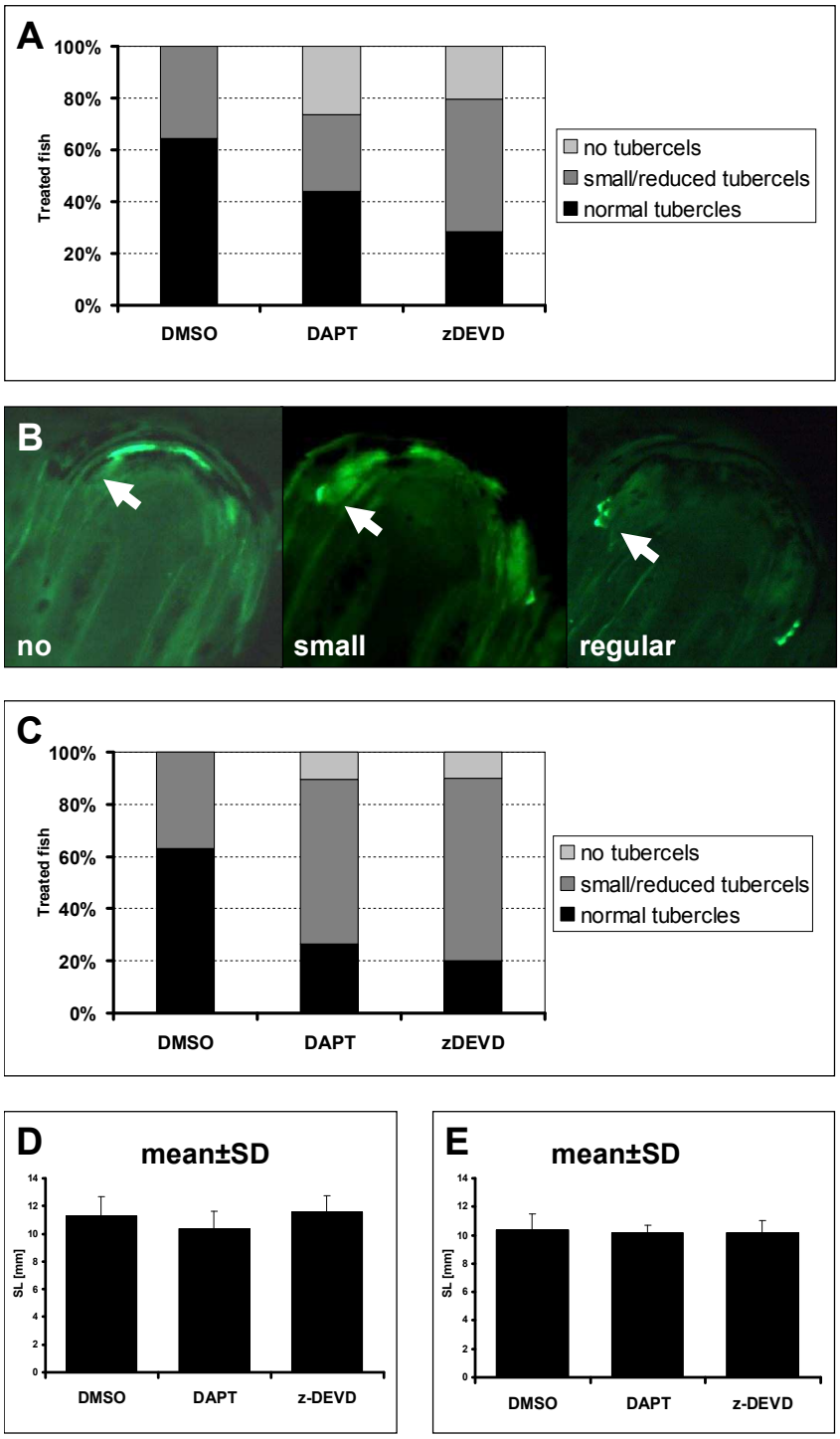

Figure 27: Inhibition of Notch signalling or Caspase 3 blocks breeding tubercle formation. Zebrafish juveniles were treated with the chemical inhibitors DAPT (γ-secreatse inhibitor) or zDEVD (Caspase 3 inhibitor) during the developmental period of initial breeding tubercle formation. (A) Formation of tubercles is reduced after treatment of both inhibitors in a three weeks trial. (B) Examples for staging: no – breeding tubercles are completely absent after the experiment; small – only very few and small tubercles had developed; regular – fish present with tubercles of wildtype size and number. (C) Formation of tubercles is reduced after treatment of both inhibitors in a four weeks trial. (D/E) SL (standard length) is measured to demonstrate equal development in treated and control groups, standard length. Both treated group display similar SL than the DMSO control group. D – SL of three weeks trial; E – SL of four weeks trial. Numbers of fish per group are listed in table 5.

Other factors might possibly contribute to this process or compensate the loss of Notch and Caspase 3 respectively. Additionally, it was not estimated, if the inhibitors had blocked their targets completely in the performed assay.

Importantly, immunostaining using the active Caspase 3 antibody demonstrated that cleaved Caspase 3 was present and active in the breeding tubercles of DMSO control fish. However, Caspase 3 did not seem to be activated when the Notch pathway or Caspase 3 itself was blocked (**figure 28B**).

Accordingly, Notch signalling activates Caspase 3 in the upper layers of breeding tubercles, which then might induce keratinocyte differentiation in an non-apoptotic way.

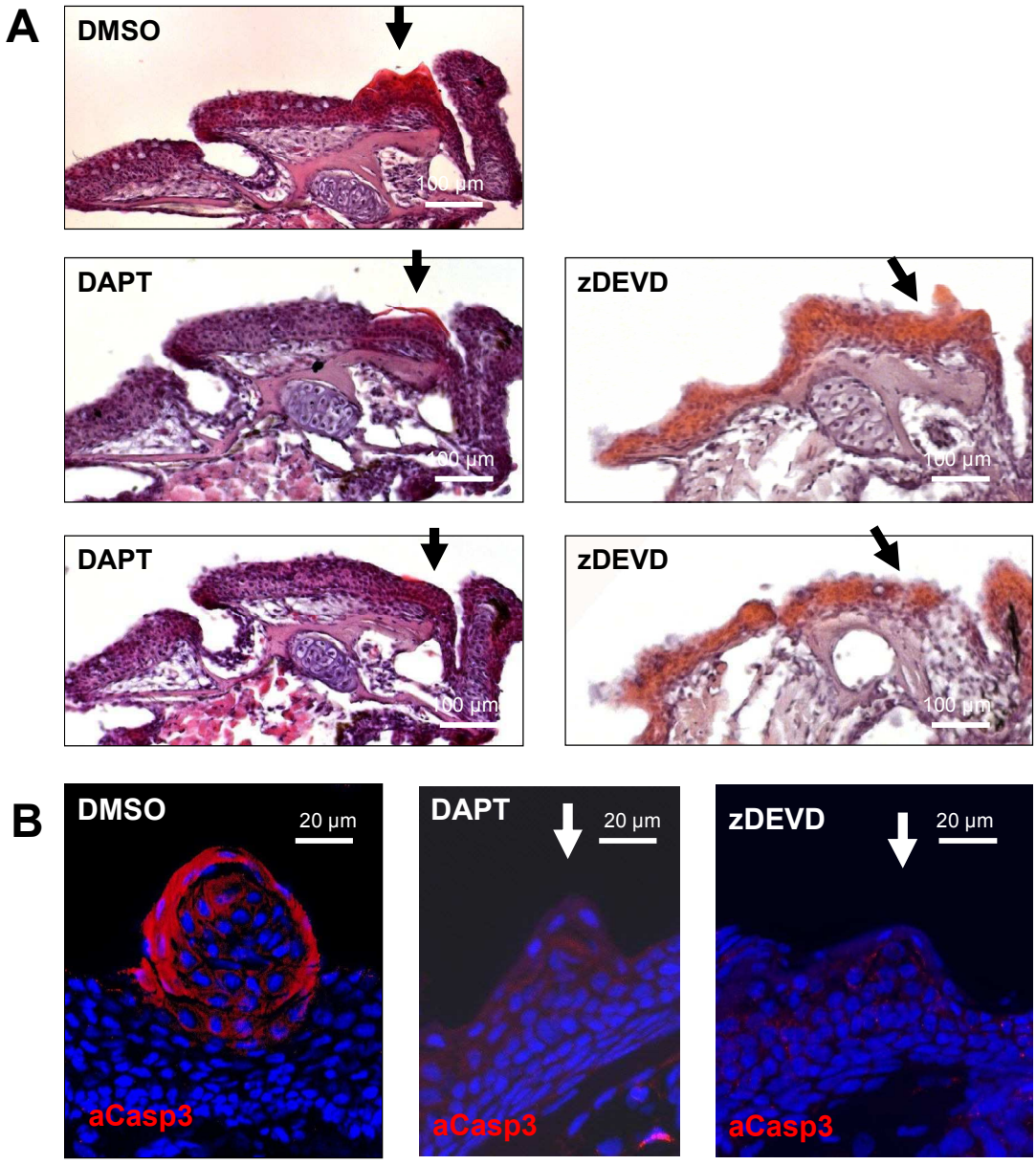

Figure 28: Inhibition of breeding tubercle formation by DAPT or zDEVD are both accompanied with loss of active Caspase 3. (A) HE staining of the tubercle region at the lower jaw of inhibitor treated fish reveales loss or reduction of tubercles. (B) Active caspase 3 is absent after inhibibion of Casaspe 3, and after inhibition of Notch signalling as well, indicating that Caspase 3 is regulated by Notch.

As we did not obtain valuable results with various *notch* and *jagged* RNA antisense probes, we used a transgenic notch signalling reporter line, *tp1bglob::GFP*^{um13}, to study Notch signalling in zebrafish breeding tubercles. In this line, binding of the RBP-J/NICD complex to the 12 RBP-J binding sites in the promoter region of the transgene drives expression of GFP (**figure 29A**) [Parsons et al. 2009]. This line was kindly provided from the laboratories of Wiebke Herzog and Arndt Siekmann (Max-Planck-Institute for Molecular Biomedicine, Münster).

GFP fluorescence could easily be detected in breeding tubercles during adulthood, as well as during tubercle development. Transgenic fish were either used directly for whole-mount imaging or prepared for cryo-sectioning. Immunostaining for GFP was not performed (**figures 29B-29E**). Importantly, Notch signalling was active only in the upper layers of the tubercle, whereas basal layer and lower spinous layer remained GFP negative. This would indicate that Notch signalling occurs only in those layers of the tubercles, where cells do not proliferate anymore and started to differentiate.

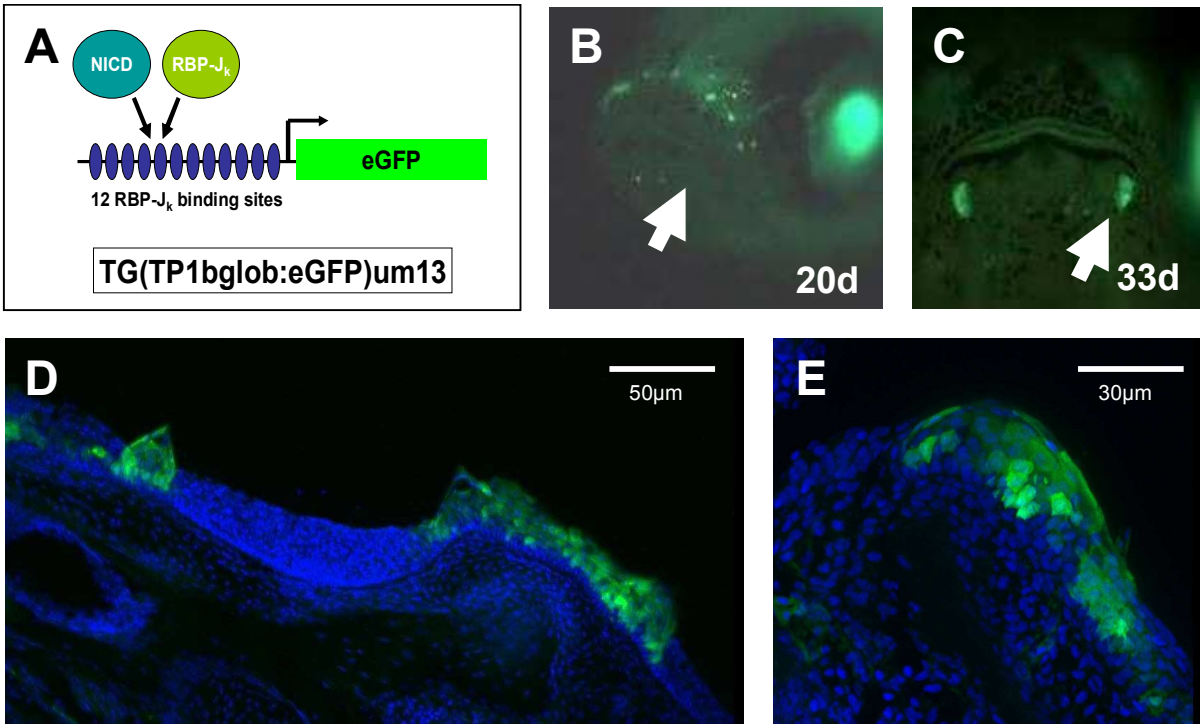

Figure 29: High levels of Notch signalling are found in the upper layers of breeding tubercles. The transgenic line *tp1bglob::GFPum13* was used to detect Notch signalling in breeding tubercles. (A) Transgene of *tp1bglob::GFPum13.* (B) Whole-mount image of a zebrafish head prior to breeding tubercle formation. No Notch signalling is detected in the area where later the tubercles will develop (white arrow). (B) Whole-mount image of a zebrafish head after breeding tubercle formation is completed. The cells of the tubercles are highly GFP positive, indicating activation of the transgene. (D/E) Sections of breeding tubercles and the surrounding epidermis. Only the upper layers of breeding tubercels are GFP positive.

Additionally, this result was confirmed by immunostaining with the anti-p63 antibody or the anti-active Caspase 3 antibody respectively. The GFP positive domain in *tp1bglob::GFP*^{um13} did not overlap with p63 positive cells, indicating that Notch signalling is not active in cells that still have the capacity to proliferate (**figure 30A**). However, overlap of a few p63 positive cells at the border of the GFP positive domain might indicate that Notch is turned on directly after or already during the switch to cell cycle arrest.

Furthermore, the domain of active Caspase 3 was completely identical with the GFP positive domain of the transgene (**figure 30B**), confirming the suggestion that Notch signalling and Caspase 3 are needed for differentiation. According to the literature, we suggested that Notch would activate Caspase 3.

To test whether Caspase 3 is indeed upstream of Notch signalling, epistasis analysis with the above described small inhibitor compounds, DAPT and zDEVD, was performed in transgenic *tp1bglob::GFP*^{um13} fish. As proof of concept, Notch signalling should be blocked in the transgenic line only in the DAPT treated group, whereas it should still be present in the zDEVD treated group if Caspase 3 is indeed upstream of Notch.

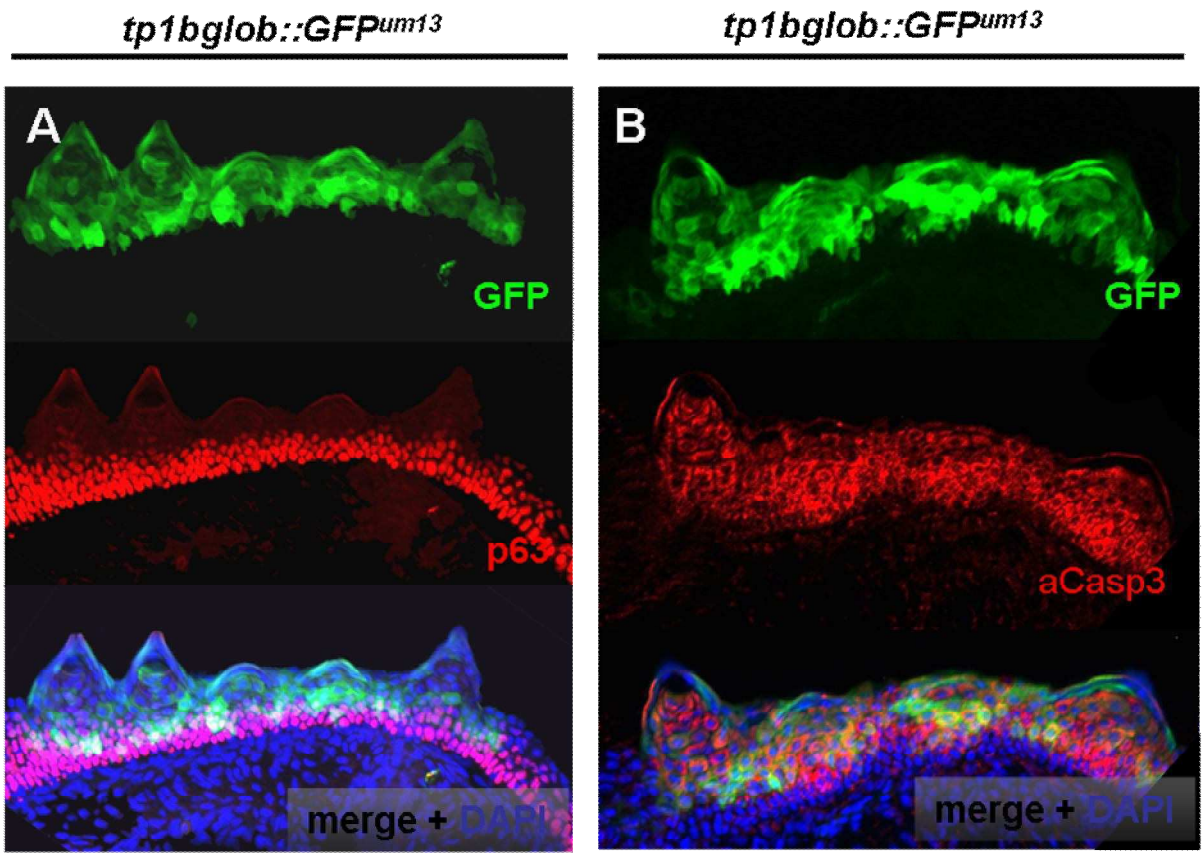

Figure 30: Notch signalling is active only in differentiating cells of the breeding tubercle. (A) p63 positive cells that still have the capacity to proliferate are below the GFP positive domain in *tp1bglob::GFPum13* transgenics. (B) Cells that are GFP positve in the transgenic line are additionally positve for active Caspase 3.

Treatment was performed over four weeks in the same way described above. Notch signalling was evaluated by whole-mount imaging and after sectioning. After the treatment, seven of seven fish in the DMSO control group displayed GFP positive tubercles. In the DAPT treated group, only one of eight fish showed fully developed tubercles. However, GFP was not turned on in this fish. Additionally, the seven remaining individuals of this group presented with no or very weak fluorescence (five without, two with weak fluorescence) in the area where breeding tubercles would develop (12.75%

present tubercles, 25% weakly GFP positive). On the other hand, the zDEVD treated group showed tubercle formation in two out of five fish (40% present tubercles), whereas Notch signalling was turned on the region of tubercle formation in all five (100%) specimens. The results are summarized in **figures 31A & 31C** Again, measurement of the standard length of all individuals revealed comparable development between all three groups (**figure 31B**).

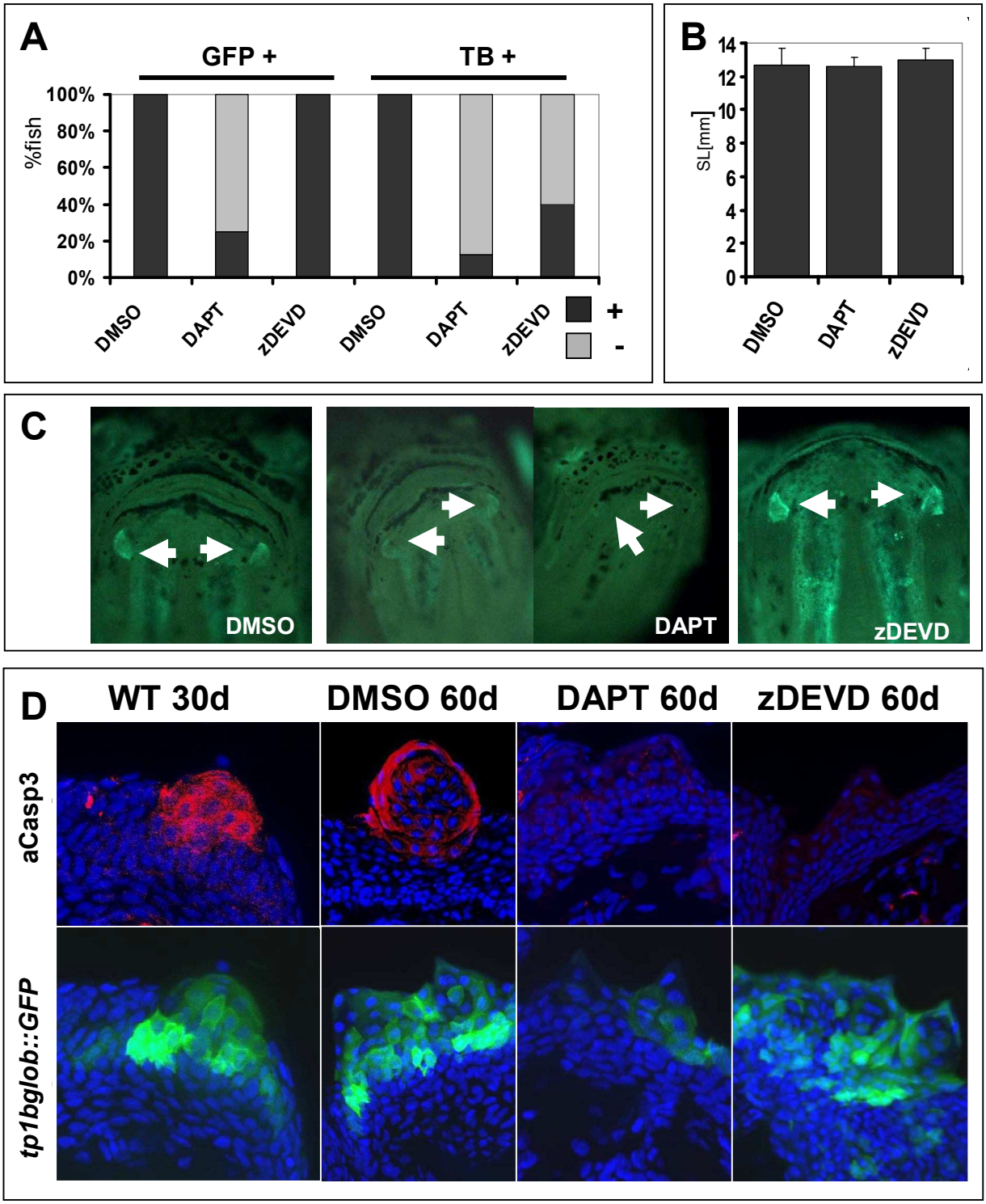

Figure 31: Áctive Caspase 3 is downstream of Notch signalling. (A) Notch signalling after inhibitor treatment with DAPT or zDEVD in *tp1bglob::GFP*. The percentage of fish with developed tubercels and with GFP positive cells in the area of breeding tubercels is shown (DMSO n=7; DAPT n=8; zDEVD n=5). (B) Measurement of standard length reveals similar developmental progress in all groups (mean±SD). (D) Examples of fish after inhibitor treatment (white arrow points to the tubercle region at the lower jaw). (D) Immunostaining of inhibitor treated transgenic fish with/without subsequent active Caspase 3 antibody staining. Additionally, young juvenile fish during initial tubercle development (30dpf) were included in this study.

Additionally, cryo-sections of treated juveniles also demonstrated that Notch signalling was blocked only in the DAPT treated group, whereas it iwas still active in the zDEVD treated group. Activation of Caspase 3 however was absent in both treated groups. To demonstrate that Notch signalling and Caspase 3 are activated in breeding tubercles already during initial development of breeding tubercles, 30 day old *tp1bglob::GFP*^{um13} transgenics were analysed for GFP and active Caspase 3. Together with the results of the epistasis analyses these data are presented in **figure 31D**.
In summary, these data indicate that active Caspase 3 is involved in keratinocyte differentiation in breeding tubercles and is induced through notch signalling.

Notch signalling is activated in immature tubercles before cap layer formation starts

To further demonstrate that Notch signalling in fact drives tubercle formation, the transgenic notch signalling reporter line *tp1bglob::GFP*^{um13} was exploited for time course studies. We claimed that Notch signalling had to be activated prior to formation of the cap layer of initial tubercles if the proposed Notch → Caspase 3 pathway drives differentiation and eventually cap layer formation in breeding tubercles.
Transgenic juveniles were examined by whole-mount imaging for onset of GFP expression in the respective areas to detect the start of Notch signalling. Additionally, calcein blue was used to specifically stain the cells of the cap layer. Calcein blue stains cap layer cells in the same way as calcein (-green). As shown in **figure 32**, Notch signalling was turned on at approximately 24 dpf (SL 7 mm). At that time, immature tubercles had already formed, but a cap layer was not detected. However, three days layer, GFP intensity had had remarkably increased and a fully developed cap layer was visible that grow in size over the next days (SL 9 mm). Altogether these results strongly point toward an important function of Notch signalling in breeding tubercle formation. Additionally, the Notch → active Caspase 3 pathway seems to be necessary for the differentiation pathway in tubercles after cells are withdrawn from the cell cycle.

Tubercle formation is compromised in TAp63 and p53 zebrafish mutants

According to evidence from mammalian epidermis, TAp63 is a potential regulator of keratinocyte differentiation [Truong et al. 2007, Truong et al. 2006, Mack et al. 2005]. TAp63 but not ΔNp63 was considered to activate Notch signalling and thereby promote differentiation via non-apoptotic functions of Caspase 3 [Mack et al. 2005]. Additionally, TAp63 is expected to play an important role in the commitment to stratification during embryonic development of the epidermis [Koster et al. 2004]. Furthermore, Jagged1 has been shown to be a direct target of TAp63 [Sasaki et al. 2002], indicating that there is a direct link between TAp63 and Notch signalling in epidermal development and keratinocyte differentiation. However, these data are still controversially discussed, especially because TAp63 specific knock-out mice do not reproduce the severe phenotype of total-p63 KO mice

[Vanbokhoven et al. 2011]. We wondered if the zebrafish as a powerful tool to generate mutant and transgenic lines might recapitulate the above described pathway.

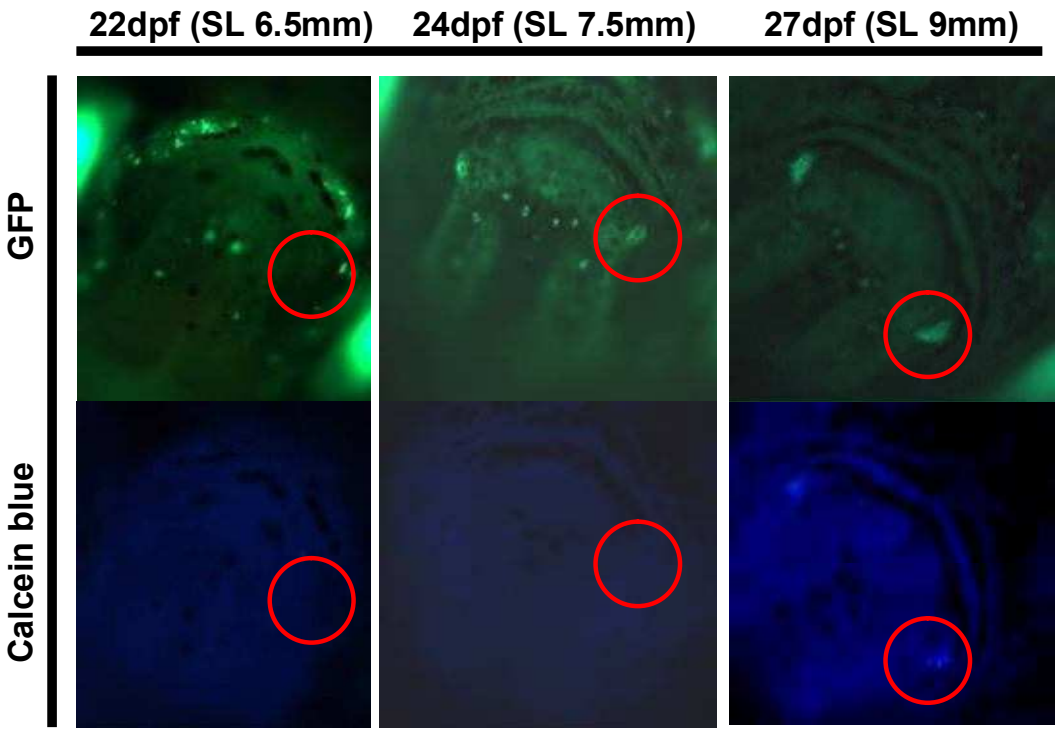

Figure 32: Notch signalling precedes cap layer formation in breeding tubercles. During initial development of breeding tubercles the formation of the cap layer was visualized by calceine blue. At 22 dpf (SL 6.5mm), neither GFP positive cells nor a cap layer are found. Later at 24 dpf (SL 7.5 mm) the tubercles are present but only in an immature state, as a cap layer has still not developed (calcein blue). At 27 dpf (SL 9.0 mm) the cap layer has formed on top of the tubercle. This finding suggests that Notch signalling is present prior to cap development.

TAp63 isoforms have not been described in zebrafish so far. All available data concerning p63 in zebrafish regard to functions of the ΔN isoforms [Bakkers et al. 2002, Lee & Kimelman 2002]. However, exon prediction in the genomic region upstream of the ΔN-specific exon (ENSDARG00000044356) and subsequent RT-PCR analysis identified two TAp63 isoforms (TA1 and TA4) that are highly similar to the identified isoforms in mammals [Bamberger et al. 2001] (**figures 33A & 33B**). The primer sequences of the TA or ΔN specific primer used for isoform specific RT-PCR analysis are listed in the Material & Methods section.

Consistent with data from mouse embryos [Laurikkala et al. 2006], TAp63 isoforms were found to be absent in early zebrafish development whereas the ΔN isoforms were already expressed (**figure 33C**). At 24 hpf, (during the initial steps of epidermal development) the shorter TA4 isoform was detected as first TA isoform, whereas the longer TA1 isoform was still not expressed. At the time metamorphosis was initiated and slightly before development of the breeding tubercles took place (20dpf), additionally the TA1 isoform was found to be expressed, suggesting a potential role of the TA isoforms in breeding tubercle formation. These results might indicate that additionally to the ΔN isoform, also the TA isoforms are needed for early epidermal development and especially during

development of breeding tubercles, where the epidermis adopts a more stratified status as regular zebrafish epidermis and the keratinocytes undergo a more advanced differentiation program.

In adult zebrafish TA isoforms were found to be expressed in the epidermis and in the ovaries, whereas they were not expressed in brain tissue. The ΔN isoform was present in all tissues (**figure 33D**). These data are in accordance with results from Suh et al., where TAp63 in mice was detected to be strongly expressed in oocytes [Suh et al. 2006].

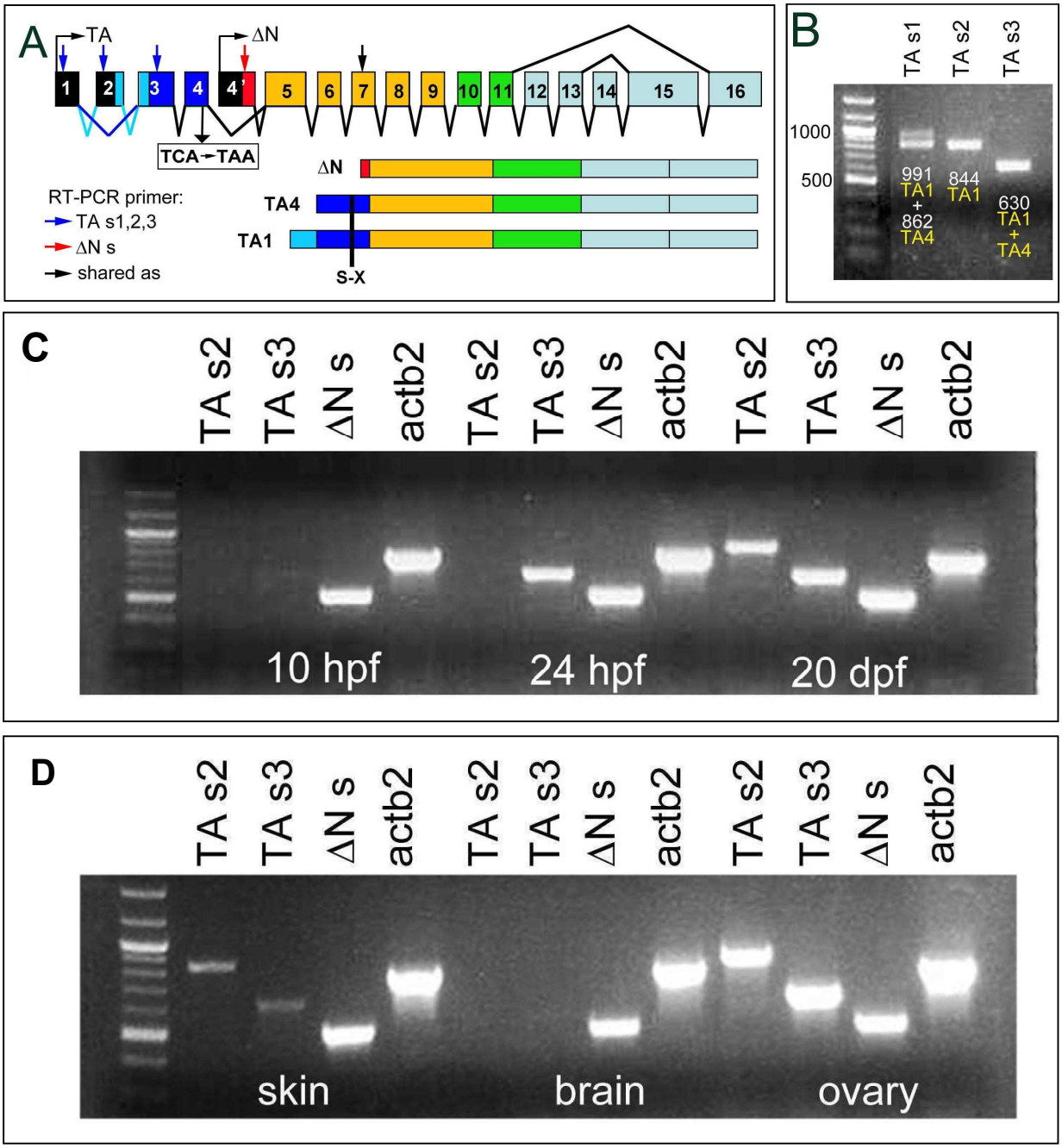

Figure 33: TAp63 isoforms and their expression pattern in zebrafish. (A) Genomic structure of *tp63* in zebrafish.The binding sites of the TA and ΔN specific primer used in this thesis are highlighted. Additionally, the predicted splice variants at the 3'- and 5'-end are shown, as well as the protein domains and the location of the mutated triplet in *tp63*^{hu2525}. Color code: blue: 5'-transactivation domain; yellow: DNA binding domain; yellow: oligomerization domain. (B) RT-PCR analysis of different TAp63 isoforms in total RNA of adult zebrafish. The localization of the different TA specific primer is shown in A. The length of the different isoforms are noted. (C) RT-PCR analysis of TA and ΔN isoforms at different stages of development.(D) RT-PCR analysis of TA and ΔN isoforms in different organs of adult zebrafish. For all experiments, a β-actin2 (actb2) fragment was used as control.

A TA-specific p63 zebrafish mutant (*Tp63*^{hu2525} allele) had already been generated using target-selected mutagenesis [Wienholds et al. 2003]. The *hu2525* allele bears a nonsense mutation (TCA > TAA) in exon 4, the last TA-specific exon, changing a serine amino acid triplet into a premature stop codon (S48X) (**figure 34**). Thereby, all TA isoforms should be abrogated in this mutant, whereas the ΔN isoforms should be not affected. The mutants were found to be viable and fertile.

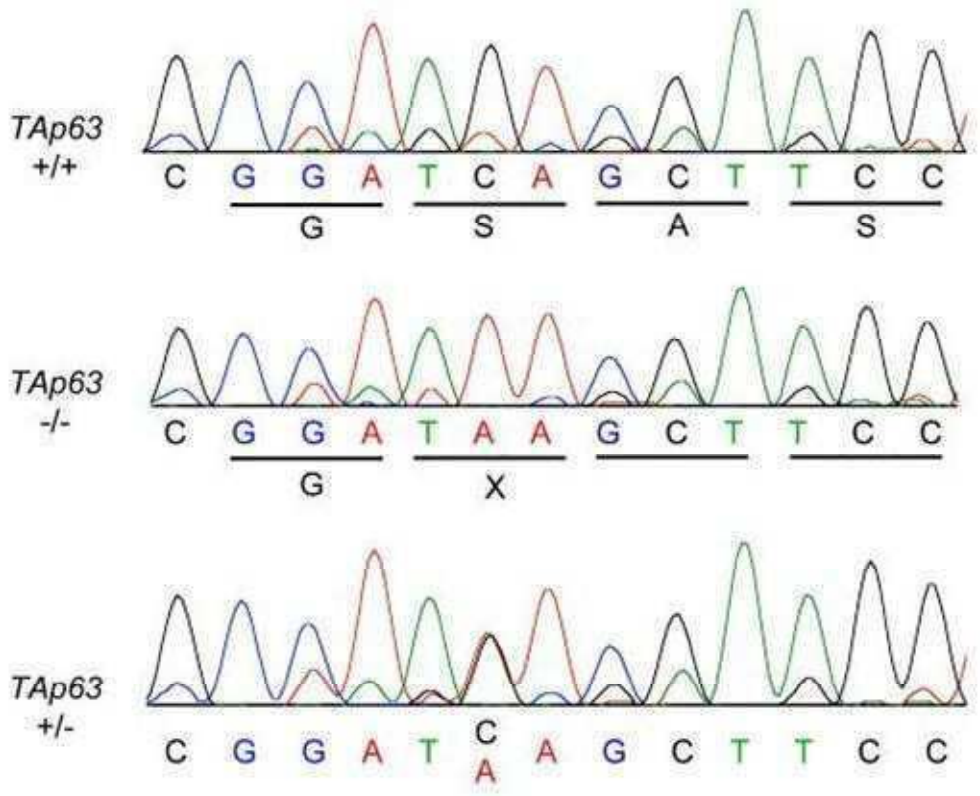

Figure 34: Mutation in tp63^{hu2525}. Chromatograph after sequencing of wildtype, heterozygous TAp63+/- and homozygous TAp63-/- fish.

As already mentioned, the three members of the p53 transcription factor family (p53, p63 and p73) share high sequence and structural similarity, especially in the DNA binding domain [Yang et al. 2010, Dötsch et al. 2010]. Binding site analysis revealed that the p53 family members bind to quite similar target site sequences in the promoter regions of target genes. Therefore, the members of the p53 family are expected to share a quite large intersection of target genes [Barbieri & Pietenpol 2005]. However, newer data also indicate unique features of the binding sites of each member [Yang et al., 2010, Perez et al. 2007, Perez & Pietenpol 2007], indicating that the different members have common targets as well as specific targets [Barbieri & Pietenpol 2005].

On the other side, the high sequence similarity of the DNA binding domain, might allow p63 and p73 to transactivate p53 target genes via the p53-responsive element (p53-RE) and vice versa [Murray-Zmijewski et al. 2006]. In particular p63 was found to regulate p53-target genes and lead to cell-cycle arrest and apoptosis. In a similar study, contrary effects were detected for the TAp63 and the ΔNp63

isoforms [Dohn et al. 2001]. Similar as for p53, a specific p63-RE exists as well, which is needed for the activation of p63 specific target genes [Perez et al. 2007].
p53 has to form homo-tetramers before it is able to recognize the p53-REs of its target genes. The same is considered for target activation by p63. It was suggested that activation of common target genes by p53 and p63 might be due to formation of hetero-tetramers. Even if yeast-two-hybrid data could not confirm this hypothesis [Yang et al. 2010, Joerger et al. 2009, Levrero et al. 2000, Davidson et al. 1999], there are also data available that promote the existence of an certain interplay: in mammalian epidermis, the start of differentiation of keratinocytes was shown to depend on RUNX1 activity, which blocks proliferation and activates the expression of Keratin 1. RUNX1 itself is activated via ΔNp63 in a p53-dependent manner [Masse et al. 2012].
In accordance with these findings, it was shown that not only p63, but also p53 can activate Notch signalling in epithelial cells. A p53-RE was detected in the promoter region of p53. In a recent publication, p53 was therefore not only linked to proliferation, but also to differentiation. According to the author's hypothesis based on *in vitro* and *in vivo* results, p53 induces growth suppression and differentiation by activation of Notch1 in keratinocytes [Yugawa et al. 2007].

To analyse this contradictory situation in zebrafish we took advantage of p53 mutant zebrafish, which are viable and fertile but frequently develop tumors during adulthood. The used *Tp53* ^{zdf1} allele bears an M214K exchange of a conserved amino acid residue in the DNA-binding domain that inactivates p53 [Berghmans et al. 2005]. We wondered if tubercle formation is compromised in the TA-specific p63 mutants and if the mutation affects the Notch → Caspase 3 pathway. According to the above described data, we decided to test p53 mutants as well.

p63 immunostaining of regular epidermis of adult mutants was not altered compared to wildtype fish (**figure 35A**), suggesting that ΔNp63 is the predominant isoform in the epidermis. This was also consistent with the RT-PCR data presented above and the previously described data from mice [Laurikkala et al. 2006].
However, breeding tubercle formation was clearly compromised in TAp63 mutant zebrafish and p53 mutants as well. Even if the strength of this effect was variable in individual fish, individuals of both mutants were detected that lacked tubercles completely and others where number and size were dramatically reduced. These results were demonstrated by calcein staining, histological staining and SEM analysis (**figures 35B-35C & 36**). However, individuals with rather normally developed tubercles were also found in both groups.
HE analysis of TAp63 and p53 mutants revealed that tubercle formation was reduced or absent in most mutants (**figures 36A-36F**). Additionally, a clearly developed cap layer could not be detected in all fish (**figure 36E**). SEM imaging also confirmed the mutant phenotype. Even if the tubercle plate structure was present in both mutants, the structure appeared clearly smaller with fewer tubercles. Additionally, the dorsal row of tubercles at the lower jaw was absent in both mutants (**figures 36G-36I**).

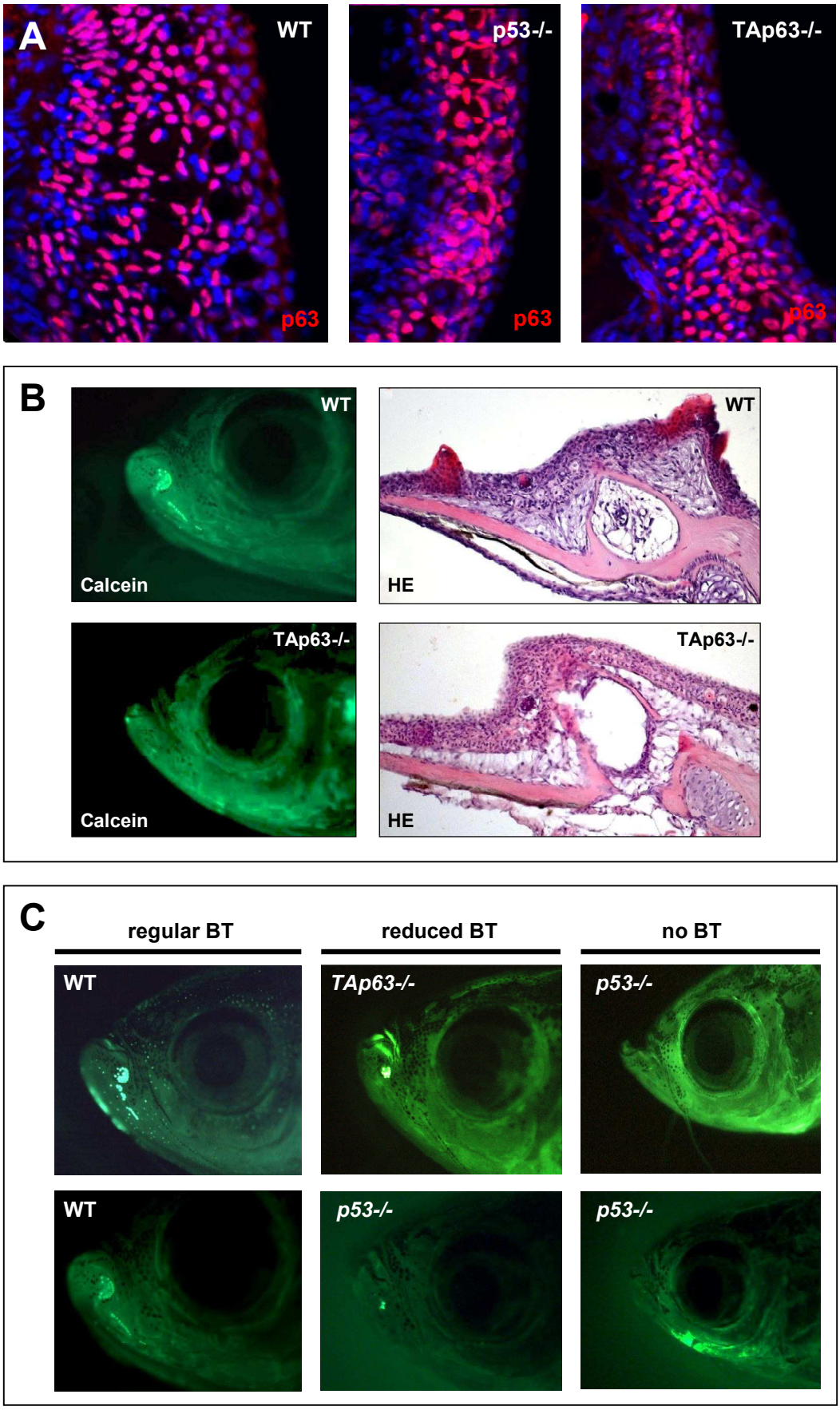

Figure 35: Breeding tubercle developmet is compromised in *TAp63* mutant as well as *p53* mutant zebrafish. (A) p63 immunostaining of regular epidermis in wildtype fish and *TAp63* as well as *p53* mutants. The distribution of potential proliferative cells is not altered in both mutants. (B) Many *TAp63* mutants display complete loss of breeding tubercels (Calcein staining & HE staining) in contrast to wildtype fish. (C) TAp63 and p53 mutant zebrafish have reduced or absent tubercles at the lower jaw (calcein stain).

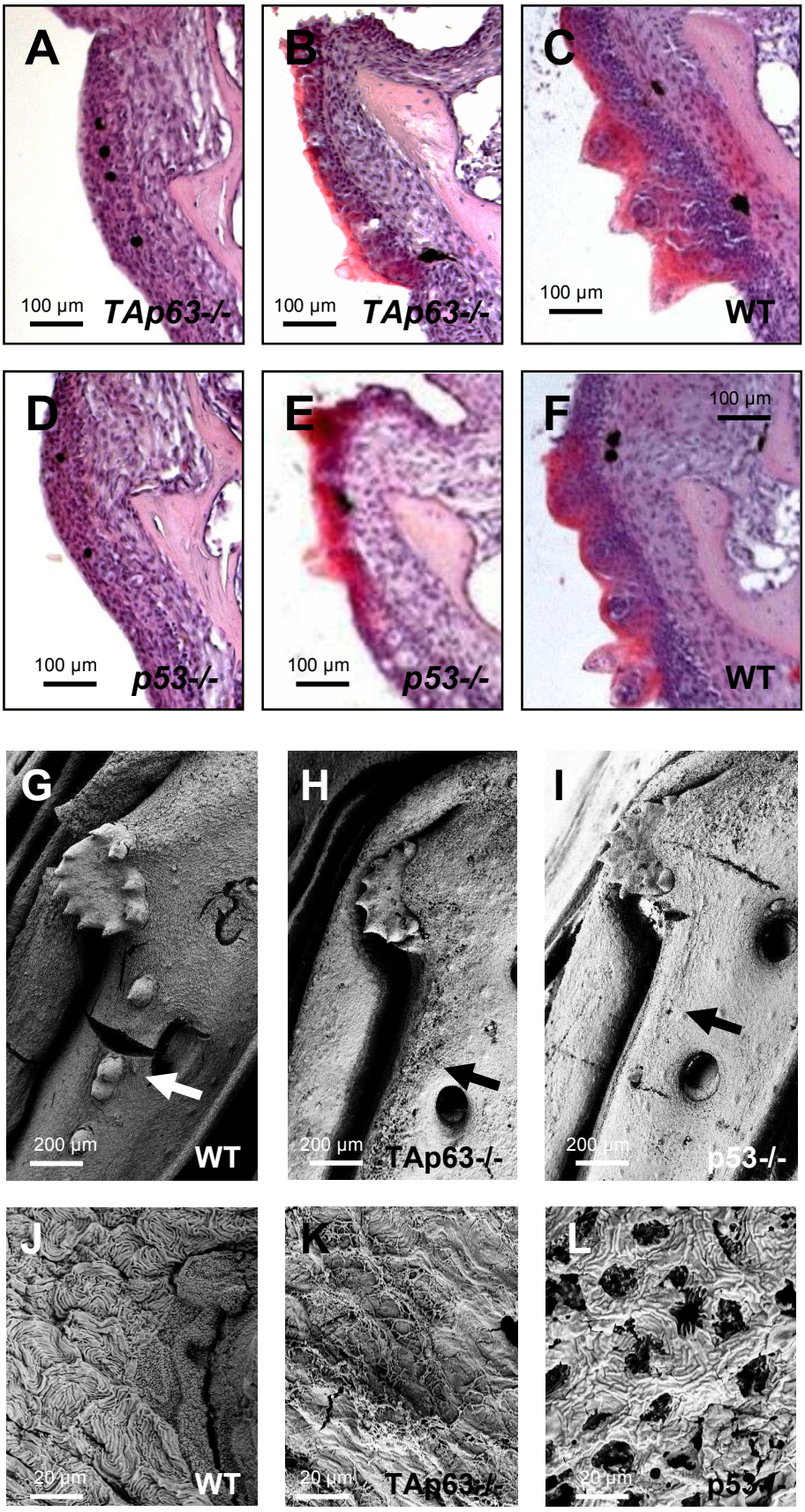

Figure 36: Phenotype of compromised breeding tubercles in adult *TAp63* and *p53* mutants. (A-F) HE staining of sections of adult wildtype, *TAp63* and *p53* mutants. For each mutant, a weak and a strong phenotype is shown. (G-L) TEM images of wildtype, *TAp63* and *p53* mutants. The upper panel displays overview images of the lower jaw region. In both mutants the tuberlce plate is smaller and the dorsal row of tubercles is completely missing (arrows). The lower panel shows superficcial layer cells of the jaw regions. Superficial cells seem to be compromised in large areas in this region.

Higher magnifications revealed that large regions of the superficial layer are compromised in TAp63 and p53 mutant zebrafish as well. In wildtype zebrafish, the regular superficial layer cells with microridges filled the regions between the tubercles at the lower jaw. However, the microridges were absent in large regions of TAp63 mutants even if no tubercles (lower jaw row) had developed. In p53 mutants, remnants of microridges could still be detected, however, large holes were found in the superficial layer, indicating that the integrity of the superficial layer was highly disturbed. Both described phenotypes were found at the lower jaw and around the tubercle plate as well (**figures 36J-36L**). However, both phenotypes of regular epidermis were not analysed in detail.

Because the number of tubercles was also very variable in wildtype fish, the exact number of tubercles in mutants was quantified and compared to wildtype fish (**figure 37A**). Mutants and wildtype fish of two different ages were analysed: approximately 50 day old fish and adult fish of 1-1.5 years. The absolute numbers of jaw tubercles per side per fish was then classified into three categories: absent tubercles, reduced number of tubercles and normal number of tubercles. The categories were assessed as follows: 50 dpf – 0-3 tubercles = no tubercles, 4-6 tubercles = reduced tubercles, 7-10 = regular tubercles; adults - <10 tubercles = no tubercles, 10-20 tubercles = reduced tubercles, >20 tubercles = regular number of tubercles. Wildtype fish presented with normal tubercle development, even if a fraction of wildtype fish displayed fewer tubercles, representing the variability in the amount of breeding tubercles. However, the majority of analysed mutants were classified as having no or remarkably reduced tubercles. This effect was more striking after 50 days of development but still present in the adult groups. For exact numbers please refer to **figure 37A**.

As tubercle formation was often only reduced in TAp63 and p53 mutants, we speculated that it might be completely abolished in TAp63/p53 double mutants, if both proteins share redundant functions. TAp63+/-; p53+/- double heterozygous individuals were in-crossed to obtain double mutant zebrafish. The genotype of 70 individuals was assessed for both mutations and yielded in 4 viable double mutant fish. However, only in two double mutants tubercle formation was not blocked completely (**figure 37A -37C**). On the other side, the effect on tubercle formation was also in these fish stronger than in the single mutant fish. This result indicated that TAp63 and p53 should have at least partially redundant functions in the formation of breeding tubercles, but other additional differentiation-promoting pathways might also be involved.

On the molecular level, the antibody against active Caspase 3 failed to detect any signal in both single mutants, indicating that both transcription factors activate the Notch → Caspase 3 pathway (**figure 38**). However, *in situ* hybridisation revealed that *tgm1* expression was remarkably reduced in TAp63 mutants, whereas the effect was rather moderate in the p53 mutant (**figure 38**). This led to the conclusion that TAp63 is the stronger activator of *tgm1* expression, or TAp63 can compensate the loss of p53. Additionally it indicated that TAp63 and p53 do not seem to have fully redundant functions in keratinocyte differentiation.

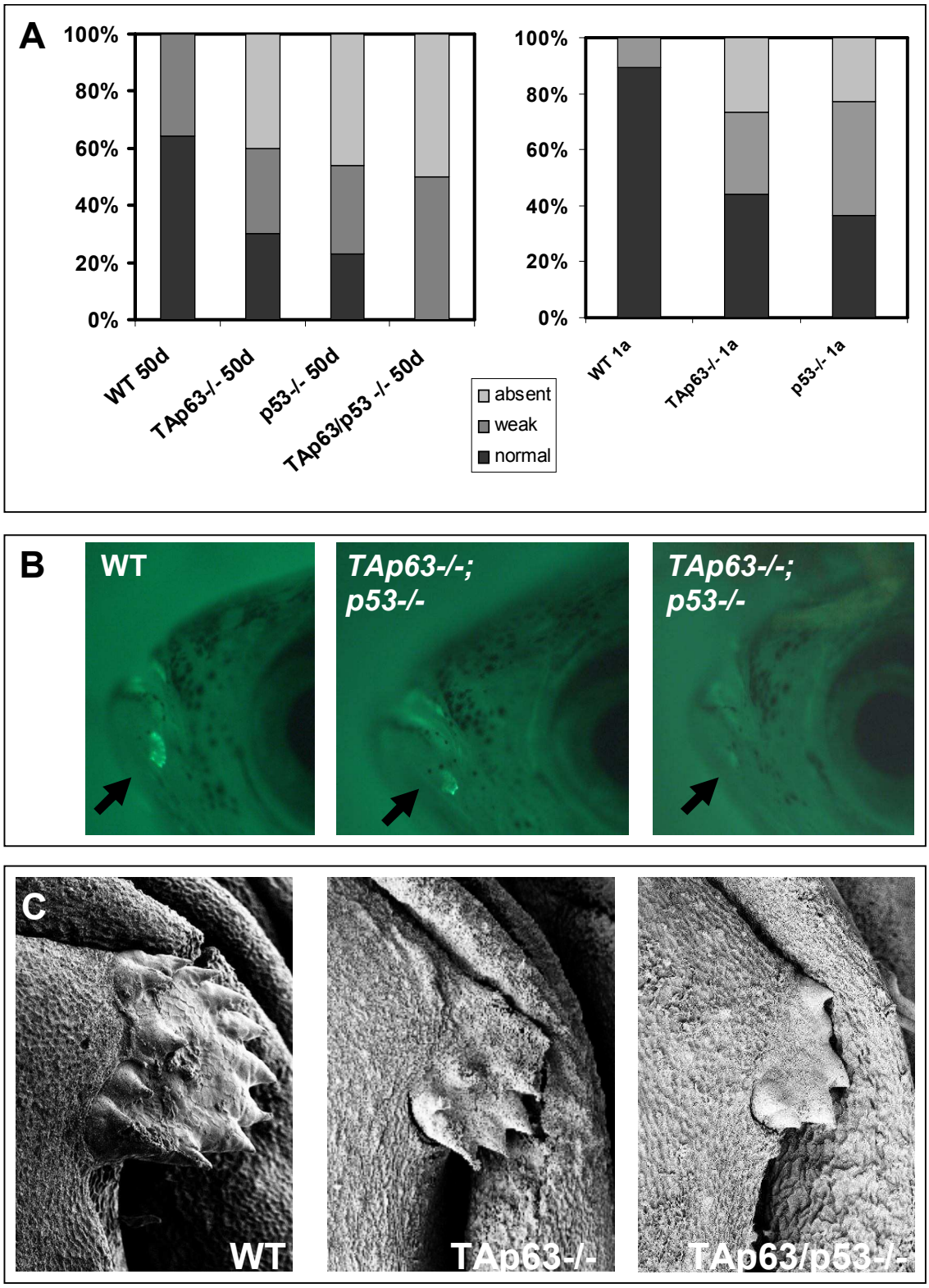

Figure 37: Breeding tubercle formation in *TAp63* and *p53* mutants is compromised throughout development and adulthood, but is still not completely abolished in double mutants . (A) Tubercle development defects at 50dpf of *TAp63-/-, p53-/-* mutants and *TAp63*;*p53*-/- double mutants, as well as wildtype control fish are presented in the left graph (wildtype n=28; *TAp63-/-* n=20; *p53-/-* n=26; *TAp53-/-,p53-/-* double mutant n=8). The right graph shows defects in breeding tubercle formation of adult *TAp63-* and *p53-* mutants in comparison to wildtype fish (wildtype n=56; *TAp63-/-* n=68; *p53-/-* n=44). (B) Calcein stain of wildtype and two double mutant heads (one with weaker and one with strong phenotype – arrows; 50 dpf) (C) Lower jaw tubercles of a WT, a *TAp63* mutant and a *TAp63/p53* double mutant zebrafish (SEM images). The compromised formation of breeding tubercels in *TAp63-/-* fish is even stronger in the double mutants, but not completely abolished (50 dpf).

These results contribute to the previously described chemical inhibitor treatment. Because breeding tubercle formation was compromised in TAp63 mutants and active Caspase 3 could not be detected anymore, we concluded that TAp63 drives Notch activation giving rise to the following pathway: TAp63 → Notch → Caspase 3. As the same results were obtained for p53, both genes seem to have at least partially redundant functions. However, the reduction of *tgm1* expression was stronger in TAp63 mutants.

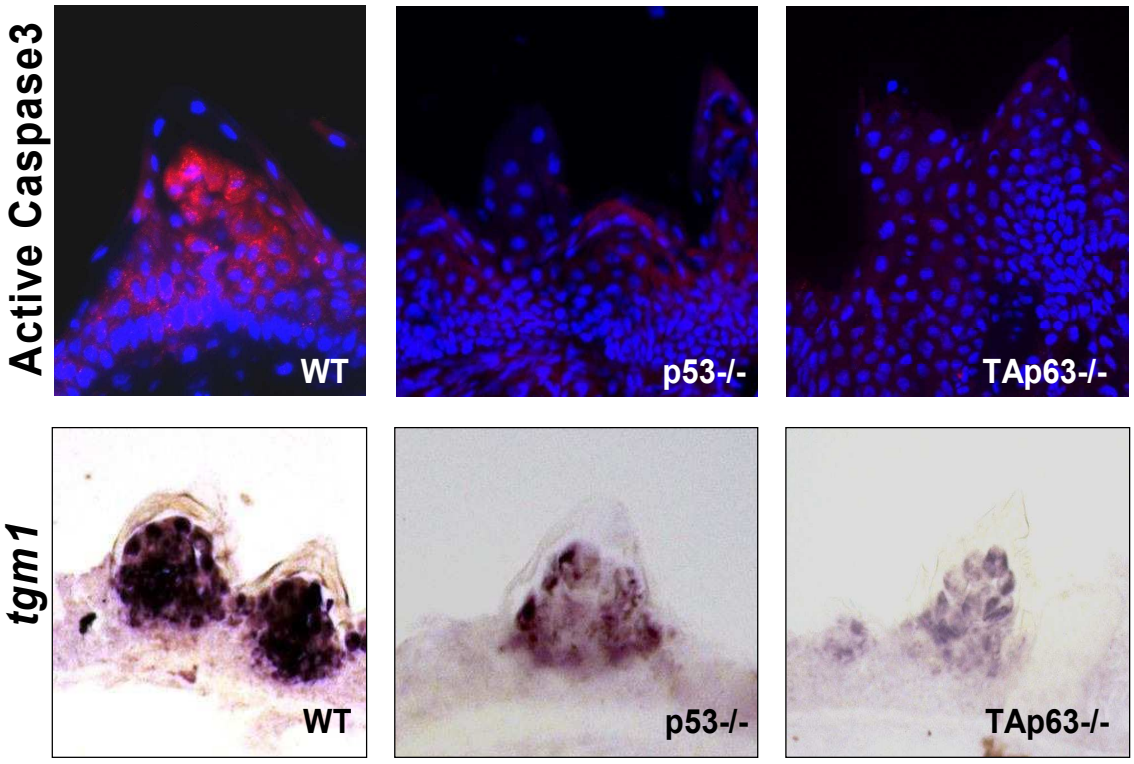

Figure 38: Caspase 3 is not activated in the compromised tubercles of *TAp63* and *p53* mutants but tgm1 expression is reduced particularly by loss of TAp63, whereas loss of p53 only has moderate effects. The upper panel displays immunostaining using the active Caspase 3 antibody. Caspase 3 is not activated in the compromised breeding tubercels od both mutants. The lower panel depicts *in situ* hybridization data of *tgm1* expression. Whereas loss of *TAp63* strongly reduces *tgm1* expression, loss of *p53* has only a more mild effect

Ectopic over-expression of Notch rescued the diminished tubercle formation in TAp63 mutants

Next we wondered how important Notch signalling might be for tubercle formation. The above described inhibitor experiments resulted mainly in a reduction and not a complete loss of tubercles. Therefore, Notch signalling might be only one of several pathways that are responsible for tubercle formation. To investigate the importance of Notch we tried to over-express Notch-ICD in juvenile zebrafish during the period of tubercle development. Practically, we took advantage of a bi-transgenic Gal4-UAS system that enabled over-expression of Notch-ICD via heat-shock [Scheer et al. 1999]. Transgenic fish of the *hsp70::gal4* line were crossed with the *uas::notchICD* line to obtain double

transgenics (**figure 38A**). Starting at 20 dpf, the juveniles were heat-shocked once per day at 40°C for one hour over four weeks. 48 individuals were assessed for both transgenes by genotyping, yielding in 8 double transgenic fish. However, Notch over-expression did not alter tubercle distribution or appearance. Additional tubercles at different locations compared to wildtypes were not detected as well as bigger or larger tubercles at the physiological sites. All transgenic fish presented like wildtype specimen (**figures 38B & 38C**). Assessment was performed with the same categories as described before.

Then we re-introduced Notch signalling into TAp63 mutants, using the same transgenic approach for temporally controlled expression of Notch-ICD as described above. TAp63 homozygous mutants were crossed to carriers of each transgene and the identified carries inter-crossed in the next generation. 137 individuals were assessed by genotyping for both transgene and homozygosity regarding the TAp63 mutation, yielding in 6 positive individuals. Despite the small number of specimens, breeding tubercle formation seemed to be restored compared to the mutants. All individuals clearly presented tubercle development that seemed to be shifted towards the wildtype condition (**figures 38C-38D**).

Together with the above described results, these data again highlight the importance of Notch signalling for tubercle formation and additionally contribute to the evidence of the TAp63 → Notch → Casapse 3 pathway.

Proliferation at the base of breeding tubercles is reduced in TAp63 and p53 mutant zebrafish

As described above proliferation is increased at the base of breeding tubercles compared to regular epidermis. A huge amount of data regarding p53 as an important tumor suppressor is available, resulting in cell cycle arrest [Hearnes & Pietenpol 2005, Levine 1997]. Interestingly, Notch was identified as a direct target of p53 in the epidermis leading to differentiation and growth arrest in cell culture [Yugawa et al. 2007]. Additionally, mammalian TAp63γ had been shown to activate $p21^{WAF/CIP1}$ via p300 and thereby inducing cell cycle arrest as well. The TAp63 function was tightly counterbalanced by ΔNp63 isoforms [Mack et al. 2005]. Therefore we wondered if loss of TAp63 or p53 protein in the respective mutants effects proliferation rates in tubercle remnants.

60 day old fish (wildtypes and mutants) were bathed in BrdU solution for 24 hours, sacrificed and sectioned for BrdU immunostaining. Three individuals were examined per group.

In all wildtype specimens, as well as in both mutants, the number of BrdU positive cells in regular epidermis was much lower than at the base of tubercles (**figures 39A-39C**). For quantitative analysis, the ratios BrdU-positive nuclei/total nuclei were evaluated (**figure 39D**). Whereas approximately 50% of all nuclei were BrdU positive in breeding tubercles of wildtype fish, the number was reduced in both mutants to approximately 35%. Interestingly, the ratio in regular epidermis was similar in wildtype fish and both mutants.

This led to the conclusion that p53 as well as TAp63 are required not only for normal differentiation of keratinocytes but additionally for proper controlled proliferation. However, the expected effect of both

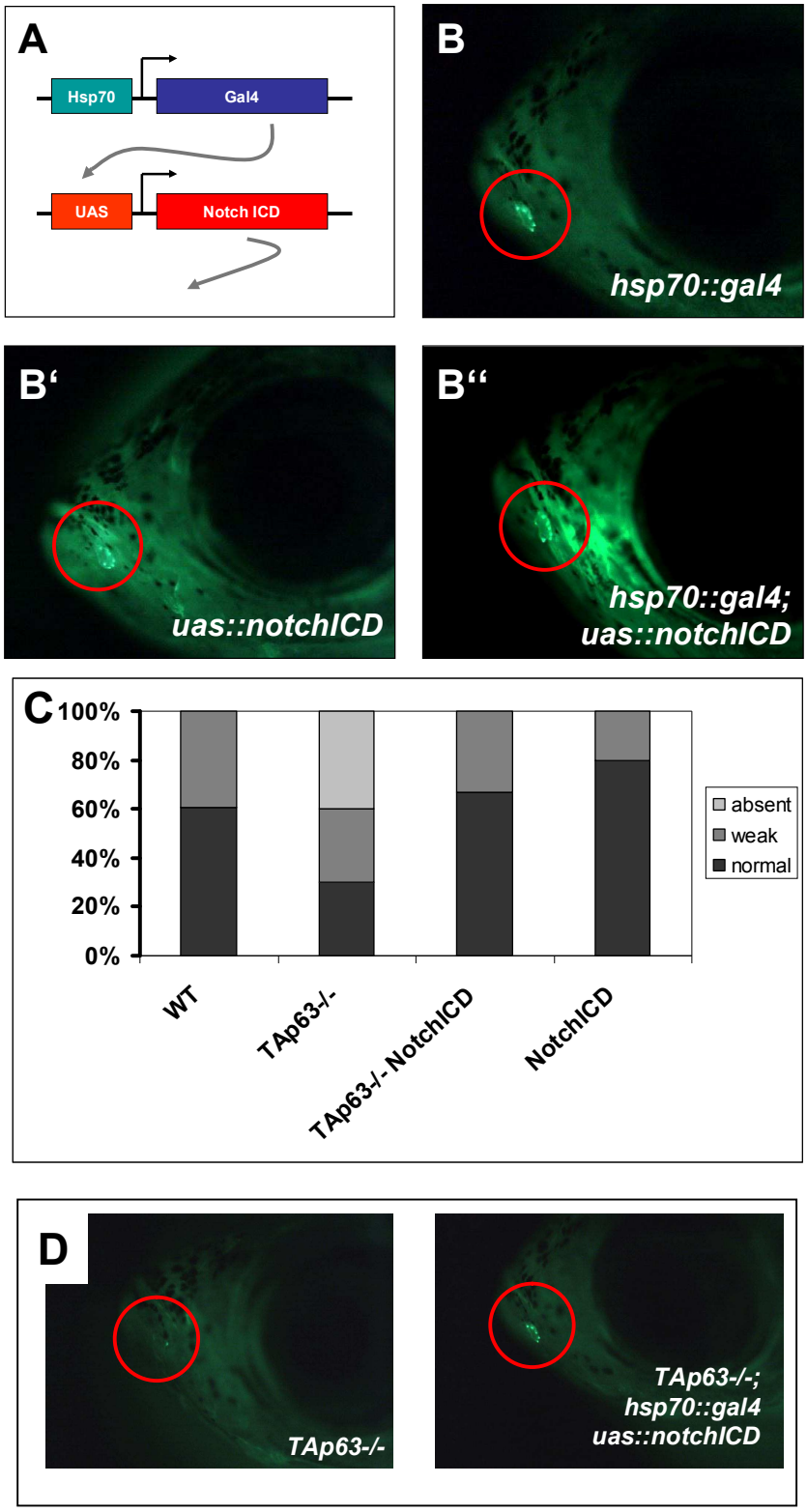

Figure 38: Reduced tubercle development in *TAp63-/-* can be rescued by ectopic notch expression. (A) Sceme of the bi-transgenic heat induceable notch over-expression system. (B) Tuberlce phenotype is not altered by ectopic notch over-expression. B - *hsp70::gal4*; B‘ – *uas::notchICD*; B‘‘ – *hsp70:gal4 x aus::notchICD*. (C) Defects in tubercle formation in *TAp63-/-* can partially be rescued by ectopic notch overexpression (wildtype n= 69; *TAp63-/-* n=10; *TAp63-/- x hsp70:gal4 x aus::notchICD* n=6; *hsp70:gal4 x aus::notchICD n=10). Categories as described.* (D) Calcein staining of head tubercles of TAp63-/- and a rescued mutant with both transgenes.

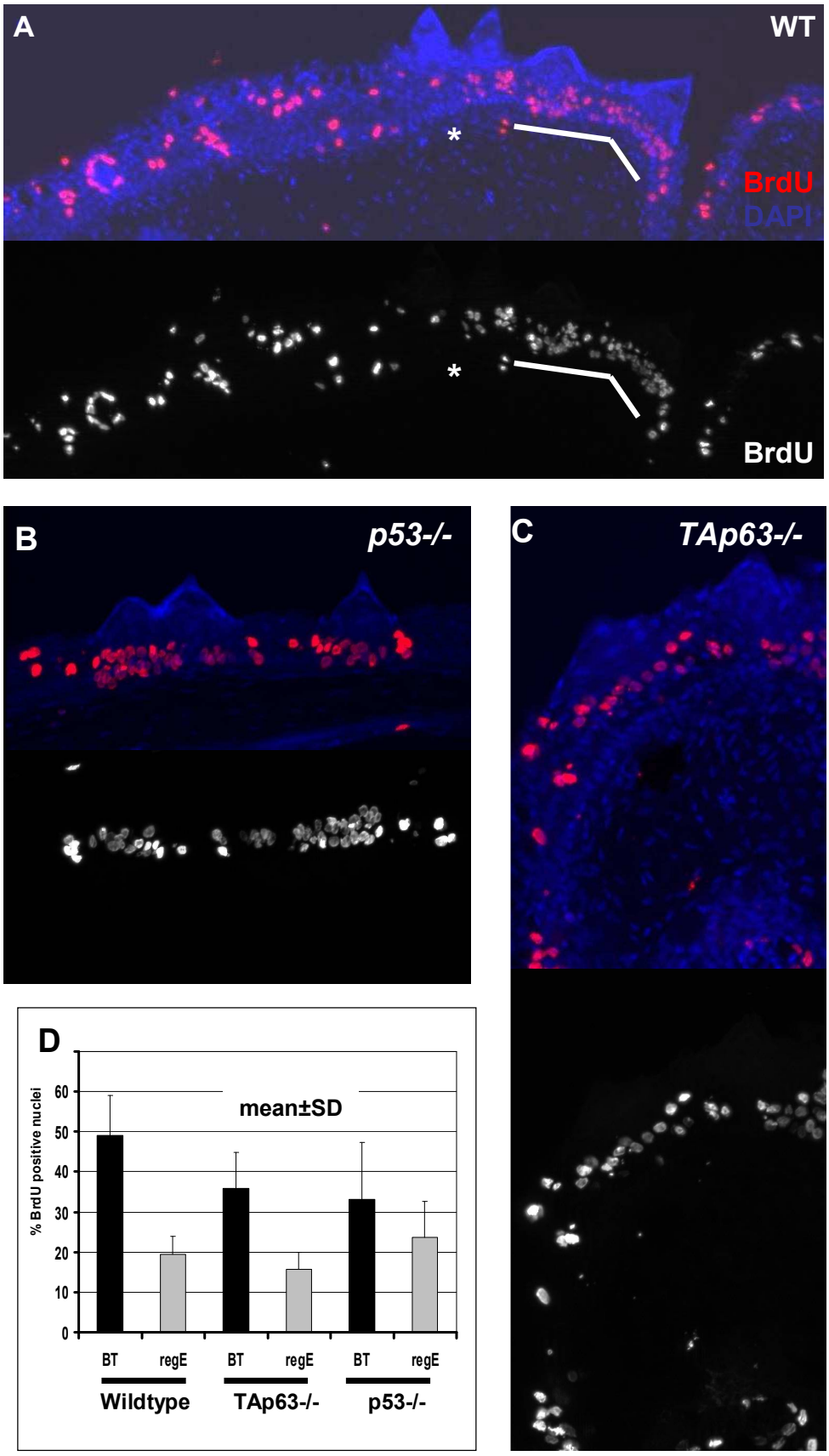

Figure39: Proliferation is reduced at the base of breeding tubercles in *TAp63-/-* and *p53-/-* mutant zebrafish. (A-C) BrdU incorporation in wildtype and mutant zebrafish tubercles and surrounding epidermis. Fish were kept in BrdU for 24 hours. BrdU positive nuclei are fewer at the base of the tubercels in both mutants. In wildtype images, regular epidermis is highlighted by asterisk and tubercle region by bars. (D) Quantification of the ratio BrdU positive nuclei/ total nuclei at the base of breeding tubercels and in surrounding epidemis; mean±SD (n=three fish each group).

regulators would have been that proliferation is increased in the mutants. The observed effect is in fact the opposite and points to a proliferation-promoting function of both proteins, which might be due to cross regulation between TAp63/p53 and ΔNp63 isoforms.

IV. Discussion

Advanced differentiation and self-renewal in breeding tubercles

Breeding tubercles in zebrafish have not been described before. However, breeding tubercles occur in many fish species especially in the cypriniformes suborder, which includes *Danio rerio*. The distribution of breeding tubercles differs dramatically among different species. In zebrafish, breeding tubercles were detected at the lower jaw and on the pectoral fins in a sexually dimorphic pattern. However, a dimorphic pattern of breeding tubercles is quite common [Wiley & Colette 1970].

In general, the following different compartments of breeding tubercles could be distinguished: a layer of basal cells, a variable number of spinous cell layers that increased in cell volume from basal to apical, a 2nd tier layer, that sealed the organism against the environment via tight junctions and the outermost cap layer of inactive cells that have lost all their organelles. Accordingly, stratification is much more pronounced in breeding tubercles than in regular fish epidermis.

It was demonstrated that the outermost layer, the cap layer, is shed regularly and renewed throughout lifetime by differentiating cells from the basal layer.

In regular epidermis proliferation occurs throughout the whole intermediate layer [Henrikson & Matoltsy 1967], as shown by p63 antibody staining and BrdU incorporation. In contrast, proliferation was found only in the cells of the lowest layers (*stratum germinativum*) in breeding tubercles. Proliferation was not detected in the upper layers of the tubercles. Additionally, the shape of spinous cells changed continuously from the basal layers to the apical surface. Differential expression of certain marker genes (like *transglutaminase 1*, various keratins and Caspase 3) indicated that the cells had started a differentiation program in breeding tubercles subsequent to withdrawal from the cell cycle. Differentiation in tubercle cells might be at least similar to terminal differentiation in mammalian keratinocytes, as important players of cornification are found to be expressed exclusively in breeding tubercles.

The differentiation process in tubercles is completed when cells of the 2nd tier layer transform into flat cap cells. This process is accompanied by loss of transcription (suggested due to the condensed nucleus and the absence of any signal in the cap layer in all *in situ* hybridization experiments) and degradation of cell organelles. Shedding of cap layers was frequently observed most probably facilitated by lysis or degradation of desmosomal contacts between cap cells and 2nd tier cells as detected in many TEM samples.

So far, we have not addressed the question if cap cells are shed only when the tight junctions are properly formed in the 2nd tier. As the regeneration of the tubercles seemed be a very fast process, it might also be possible that new tight junctions are also formed after shedding. In the layers beneath the 2nd tier, no signs of tight junctions were found, indicating a unique process only in cells of the 2nd tier.

Barrier function in mammals is achieved not only by tight junctions but also by the extracellular lipid envelope. In mammalian epidermis, vesicles with lipid content are exocytosed into the extracellular space, where they form additional lipid lamellae around the cells to prevent water loss. Even if water loss is not a problem for fish, lipid lamellae were definitely found in the breeding tubercles of zebrafish. They could be detected preferentially between the cap layer and the 2nd tier cells, but also between the uppermost cells of the spinous layer. This lipid envelope was probably formed by vesicles at the basal side of cap layer cells that discharged their lipid content into the extracellular space. Interestingly, those vesicles of analogue processes in mammalian epidermis are found only at the apical side of the cells of the granular layer. The epidermis of the frog *Rana pipiens* has been shown to be capable to form keratin containing granules and mucous granules as well in differentiating keratinocytes [Parakkal & Matoltsy 1964], which are therefore clearly different from vesicles in mammalian epidermis. The mucous granules are discharged into the extracellular space to protect the organism from dehydration due to the water-binding properties of mucus. Even if the authors do not refer to the mechanism of vesicle discharge or to a possible formation of extracellular lipid lamellae as well, the vesicles detected in the cap layer of zebrafish might resemble a more primitive form of these vesicles in the context of a cornification-like process, because the duality between mucous granules and keratin-containing granules was found only in differentiated keratinocytes [Parakkal & Matoltsy 1964]. However, the mucous granules in keratinized epidermis of *Rana pipiens* were shown to be PAS positive, what was not the case in any cells of the breeding tubercle.
In 2004, Alibardi et al. presented electron microscopy data regarding keratinisation in turtle epidermis [Alibardi et al. 2004] and showed that vesicles that contain mucus and possibly lipids discharge their content into the extracellular space. The presence of lipid/mucus granules in turtle epidermis was already described before, but depends highly on the fixation method [Henrikson & Matoltsy, 1970]. Mucus is formed in the endoplasmatic reticulum and processed in the Golgi apparatus and eventually packed into vesicles [Alibardi et al. 2004]. However, the data do not show if the vesicles fuse at the apical or basal side with the plasma membrane. Furthermore, the author points out that the intercellular material is highly electron-dense, what is not the case in zebrafish. They find these vesicles in the upper cells of the spinous layer and in the granular layer. Not all these vesicles are discharged into the intercellular space. Many of them also stay inside the cell to build up an inter-keratin matrix that mainly consists of mucus and lipids [Alibardi et al. 2004]. Probably, the vesicles seen at the basal side of the cap cells are not only needed to form and maintain a lipid envelope, but also change the intracellular arrangement of the keratin network. As seen in TEM sections, keratins filaments are orientated parallel to the cell surface in cap cells, whereas they are not detectable at all or only at desmosomes in the tiers underneath.

The cap layer reacted differently in all histological methods indicating its different composition compared to regular epidermis and the lower layers of breeding tubercles. The eosinophilic cytoplasm of this layer in hematoxylin/eosin or MGT staining might point to high amounts of keratin and/or a

keratinized character. Additionally, the pyknotic nuclei and the loss of organelles indicated that those cells are prone to a shedding process.

Even if this nuclear appearance and of course the positive reaction with the active Caspase 3 antibody might argue towards apoptosis, the cap layer nuclei were always TUNEL negative. Additionally, apoptotic cells do not completely loose their cell organelles. TEM imaging revealed that cell membranes of cap cells often appeared degraded or leaky sometimes possibly even connecting neighbour cells to a syncytium, what is never found in apoptosis. Even if the nuclei are not degraded completely, cap layer cells might resemble a sort of cells death that resembles a more primitive form of cell death during cornification.

In contrast, the superficial layer of regular epidermis clearly presented with healthy not condensed nuclei and the typical microridges at the apical surface. In contrast, microridges were never found in cap cells.

Analysis of the distribution of marker of the superficial cells revealed absence of GFP in transgenic *krt4::GFP* fish and no reaction with a S100 antibody in cap layer cells of breeding tubercles, whereas both were present in the superficial layer of the surrounding epidermis. This initially indicated a shedding process but additionally led to the suggestion that the outer cells of the superficial layer and the cap layer might resemble distinct types of differentiation in fish epidermis. However, the results of the lineage tracing analysis revealed that the superficial layer of regular epidermis in adult zebrafish consists of a mixture of cells that are derived from the original embryonic EVL and from cells of the embryonic basal layer. During adulthood, the EVL seems to be replaced step by step by cells from the basal/intermediate layer of the epidermis. In contrast to the more homogenous appearance of the epidermal cells in the intermediate layer, cells that end up in the superficial layer undergo dramatic changes in shape (flat appearance with microridges) and expression (e.g. genes of the tight junction complex). In general, the cells obtain the same features as the EVL derived cells.

Cap cells of the breeding tubercle on the other side, were found to develop exclusively from cells of the basal *stratum germinativum*. The remnants of the EVL might get lost during the first shedding cycle and in the following renewal of the cap layer by differentiation of 2nd tier cells replaces the superficial layer at the outside of breeding tubercles.

Therefore, two distinct differentiation pathways might exist in fish epidermis. One pathway is driving intermediate layer cells into the superficial layer to replace EVL cells. During this process, the epidermal cells have to undergo certain structural changes including flattening and the formation of the microridge structure. The second mechanism is more similar to processes in mammalian epidermis. Epidermal cells from the *stratum germinativum* differentiate continuously throughout the whole epidermis until the reach their final stage as a flattened cap cell and are sloughed off eventually. In comparison with mammalian embryonic development, one might conclude that regular fish epidermis resembles at least partially mammalian embryonic epidermis prior to initial cornification. At that stage, the embryo is covered by viable periderm cells that are derived from the basal layer.

Furthermore, the region of the breeding tubercles might resemble the status of initial cornification in mammalian embryos, where the periderm layer is shed and the layers underneath have originated

form the basal layer until they eventually have undergone cornification. Overall, the cells of the cap layer seem to be more similar to first "cornified" cells of fetal human epidermis that were described as "immature horny cells with nuclei". By evaluation of TEM data these cells were considered to represent a "transition phase" of keratinization" [Holbrook et al. 1975].

However, there are also clear differences to cornification of land-living animals. As already mentioned, a cornified envelope was not detected in breeding tubercles. The typical cross-linking proteins of amphibian and mammalian epidermis (like filaggrin, involucrin or loricrin) do not exist in fish genomes [Vanhoutteghem et al. 2008]. Therefore formation of a cornified envelope is not very likely. However, the strong expression of the cross-linking enzyme Transglutamiase 1 in the upper layers of the breeding tubercle might indicate that similar substrates exist in fish. Additionally, the nucleus of cornified cells gets completely lost, whereas it was still present (even if in a pyknotic and inactive form) in breeding tubercles.
However, also the epidermis of water-living mammals as for example whales adopts an at-least "fish-like" character. The epidermis of whales is still cornified but in a parakeratotic manner, meaning that the outer cornified layer includes a remarkable amount of living cells [Reeb et al. 2007].
In mammalian and amphibian epidermis tight junctions are found in the *stratum granulosum* or in the outermost viable cell layer. Additionally, most steps of cornified envelope formation and lipid envelope formation occur in the granular layer. Accordingly, in a "comparison" of the epidermis of teleost breeding tubercles and regular mammalian or amphibian epidermis, the cap layer would resemble the *stratum corneum* of tetrapod epidermis and the 2nd tier layer the *stratum granulosum* (**figure 40**).

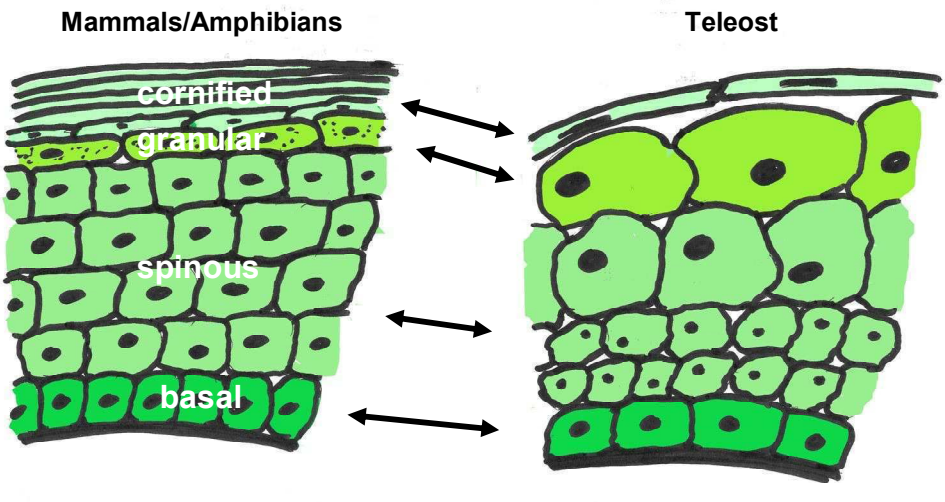

Figure 40: Comparson between mammalian/amphibian epidermis and breeding tubercels in zebrafish according to the disscussed data.

Breeding tubercles in an evolutionary context

Cornification is especially important for land-living higher vertebrates to prevent water loss and dehydration. Therefore, processes like cornification are not considered to be necessary for aqueous vertebrates. However, according to the data presented in this thesis, we concluded that at least a more primitive type of cornification does already exist in breeding tubercles of various fish species.

As already described, the differentiation process in breeding tubercles seems very different from mammalian cornification. However, land-living amphibians also display only one layer of cornified cells with an underlying 2nd tier layer that seals the organism via tight junctions. HPRs like fiaggrin are also not known in amphibians as well as a cornified envelope. Additionally, parakeratosis occurs quite frequently in amphibian epidermis. Also in amphibian epidermis specific secondary sexual organs are described on the fingers of adult male amphibians, called nuptial pads that facilitate a firm grip by males on females during egg laying [Fox 1986]. This suggests that formation of those more advanced epidermal appendages is conserved at least between fish and basal terrestrial vertebrates. However, it might also indicate that the advanced genetic system of cornification had been elaborated in the course of evolution starting from a "primitive" differentiation process in contact organs of fish. In accordance with this hypothesis would be that Notch signalling and Caspase 3, which are known to be essential regulators of mammalian cornification [Okuyama et al. 2008, Blanpain et al. 2006] are also required for formation of breeding tubercles in zebrafish.

Keratin network in zebrafish breeding tubercles

In general, the ultrastructure of breeding tubercles in zebrafish resembled the ultrastructure of elevations in Bagarius [Mittal & Whitear, 1979]. However, a few remarkable differences could be found. Hemidesmosomes were described to attach the basal layer of Bagarius to the basement membrane. In general, hemidesmosomes are connected intracellularly with the keratin cytoskeleton [Fuchs & Rhagavan, 2002] and connect the cytoskeleton to integrins at the outside of the cell. However, no signs of hemidesmosomes were found in all zebrafish TEM sections. Additionally signs of keratin filaments or tonofilaments were only scarcely found. Accordingly, immunostaining with the pan-keratin antibody gave only a weak reaction in the basal layer of the tubercle. However, desmosomes could clearly be detected between cells of the basal layer and lower spinous layer. Basal cells were always tightly connected to the basement membrane and at least during larval stages the existence of hemidesmosomes was clearly shown in zebrafish [Sonawane et al. 2005]. Therefore the absence of hemidesmosomes cannot be explained.

The most important difference between the elevations of *Bagarius* and the breeding tubercles of zebrafish was the abrupt transition of cellular character in the middle of the elevations of *Bagarius*. This transition was caused by rearrangement of the keratin filaments from a more even distribution in the lower layers into larger bundled keratin cables in the upper layers [Mittal & Whitear, 1979]. This transition was not detected in zebrafish breeding tubercles.

As keratin content in fish epidermis was controversially debated for a very long time [Padhi et al. 2006, Conrad et al. 1998, Burgess 1965], and given the evolutionary distance between fish and mammalian keratin genes, one might suggest that keratins in fish do not (or only to a minor extend) form the typical dense network as known from mammalian epidermis but exist in a more amorphous state. Additionally, keratin bundling was described in *Bagarius*, predominantly in the upper tiers of the elevations, whereas the lower tiers presented a more amorphous state of keratin distribution [Mittal & Whitear 1979]. Additionally the great increase in keratin that was indicated by immunohistochemistry with a pan-keratin antibody was not found in TEM images. On the other side, large homogenous aggregates could be seen, often in contact with desmosomes. These condensations might represent keratin aggregates that do not form fibrils. To make it even more complicated, *in situ* hybridisation with different keratin specific RNA-probes showed that some keratins are expressed only in epidermal cells at the base of the tubercle. This might indicate that keratins are expressed preferentially in those epidermal cells that are still mitotic active, but mature perhaps into specific aggregates after the cells are withdrawn from the cell cycle.

Additionally, these aggregations were not found in regular fish epidermis indicating that they might be specific for breeding tubercles. Together with the differential expression of the tested keratin *in situ* probes and the *tgm1* expression profile, one might suggest substantial differences in keratin composition and properties in breeding tubercles compared to regular fish epidermis.

Interestingly, keratin inclusion bodies are also found in apoptotic cells of mammalian epidermis. These bodies contain KIFs together with various apoptosis-mediating factors, thereby modulating different ways of apoptosis in keratinocytes. In humans keratin bodies are often associated with certain diseases (e.g. different dermatoses, lupus erythematosus or lichen planus) [Lin et al. 2008, Grubauer et al. 1986]. One might speculate that similar processes occur in cornification-like processes of breeding tubercles. In turn this would be another parallel between cornification and apoptosis.

The linear pathway TAp63 → Notch → active Caspase 3 is needed for keratinocyte differentiation in zebrafish as well as mammals

Whereas the ΔN isoforms of p63 are well know to have important functions during early steps of epidermal development, functions of the TA counterparts are still controversially discussed. ΔNp63 was shown to have important functions regarding proliferation and stemness of basal keratinocytes [Romano et al. 2012, Bakkers et al. 2002, Lee & Kimelman 2002], whereas TAp63 specific knock-out mice lack the severe phenotype of the (pan-)p63 knock out [Vanbokhoven et al. 2011]. In contrast to mutants of the canonical notch signalling partner RBP-J and Casaspe 3 mutants [Okuyama et al. 2008, Blanpain et al. 2006], TAp63 mutant mice do not have a severe epidermal phenotype at all [Su et al. 2009, Suh et al 2006]. The same is true for mutants of the more prominent member of the p53 transcription factor family, p53 itself.

In this thesis, we had shown that tubercle formation is compromised and reduced in TAp63 mutant and p53 mutant zebrafish as well. Additionally, chemical inhibition of Notch signalling and Caspase 3

as well led to the same phenotype. In accordance, active Caspase 3 levels were dramatically reduced in mutants due to inhibition of the canonical Notch pathway. Furthermore, this phenotype could be rescued by re-introducing Notch signalling into TAp63 mutants. This led to the conclusion that the linear pathway TAp63 → Notch → active Caspase 3 that was already proposed in mammals [Mack et al. 2005] to drive terminal differentiation of keratinocytes already exists in fish epidermis.
As already explained, the fact that the p63 positive domain in breeding tubercles (stained by the 4A4 pan-p63 antibody) did not overlap with the domain of Notch signalling or Caspase 3 should not be contradictory to the above proposed pathway. The antibody did not discriminate between the different isoforms and it was already described that ΔNp63 should be more abundantly expressed in the epidermis than TAp63 [Vanbokhoven et al. 2011, Koster et al. 2007]. This was also confirmed by the RT-PCR data from skin extracts and the unaltered immunofluorescence signal of p63 (4A4) in regular epidermis. This indicated that ΔNp63 is expressed in the basal layer of tubercles complementary to the Notch and Caspase 3 domain and in contrast to the broad signal in the intermediate layer in regular epidermis. As the expression pattern of TAp63 still remains unknown, one could argue that the TA isoforms might have a broader expression pattern, perhaps more similar to p53 [Pan et al. 2003], including the upper layers of the breeding tubercles.
Yang et al. already suggested that TAp63/p53 activity in maturing mouse keratinocytes is higher than in immature keratinocytes [Yang et al. 2000], which express ΔNp63. as already mentioned, the ΔN isoforms are shown to be dominant-negative regulators of TA isoforms. Taken a broader expression pattern for TAp63 into account, we believe that TAp63/p53 activity in zebrafish breeding tubercles is indirectly regulated via negative inference with ΔNp63. The ΔN isoforms, which are only expressed in the *stratum germinativum* block their TA counterparts in this layer, whereas the TA isoforms remain active in the upper layers of the tubercle.

ΔNp63 has been shown to be a direct target of Smad-mediated signalling by Bone Morphogenetic Proteins (BMPs) [Bakkers et al. 2002] In accordance with the data that BMP signalling is suppressed in skin appendages like hairs and teeth in mammals by Ectodysplasin signalling [Laurikkala et al. 2002], we speculated that the TAp63 → Notch → active Caspase 3 pathway might be regulated via Ectodysplasin, which then activates BMPs and in turn blocks ΔNp63. Consequently, breeding tubercle formation should also be compromised in mutants of Ectodysplasin signalling. As ΔNp63 would not be abrogated in the absence of Ectodysplasin signalling, the tightly controlled balance between the ΔN and TA isoforms would be disturbed. According to our model, if ΔNp63 expression ceases due to Ectodysplasin-induced transcriptional activation of BMP inhibitor genes (Noggin/Chordin [Bakkers et al, 2002]) tubercle formation should be induced.
However, *finless* and *nackt* fish (zebrafish mutant lines with complete loss of Ectodysplasin receptor and Ectodysplasin ligand respectively) [Harris et al. 2008] displayed rather normal phenotypes concerning the breeding tubercles. On the other side, a closer evaluation (e.g. counting the number of tubercles, marker analysis) was not performed so far.

In accordance with this model, Notch1 has been shown to repress ΔNp63 expression [Nguyen et al. 2006], while TAp63 induces caspase-dependent ΔNp63 degradation [Wu et al. 2011], indicating a proper controlled balance by various feed-back loops.

The p53/TAp63 → Notch → active Caspase 3 pathway might be involved in regulation of shedding and renewal in breeding tubercles

One striking result of this thesis was that loss of TAp63 and p53 as well did not only affected keratinocyte differentiation, but also proliferation at the base of breeding tubercles. Proliferation was reduced in both mutants compared to wildtype fish, as shown by BrdU incorporation, indicating dual functions (inducing differentiation and proliferation) of both regulators. In addition, the same effect was also observed by chemically inhibition of Notch and Caspase 3. Importantly, both are activated only in the upper domain of breeding tubercles, where the cells do not proliferate anymore and have become post-mitotic. This argues for a non-cell autonomous mitogenic effect of the two regulators.

Interestingly in *Drosophila melanogaster* wing discs, the caspase Dronc, which is generated in apoptotic cells upon injury, does not only execute cell-autonomous cell-death, but additionally stimulates adjacent cells non-cell autonomously to proliferate and compensate for the loss of the dead cells. This non-autonomous effect promoted proliferation, even when Dronc-positive cells were prevented from dying, indicating that a Dronc-dependent signal acts in an apoptosis-independent manner [Lamkanfi et al. 2007, Huh et al. 2004]. We suggest a similar mechanism in zebrafish breeding tubercles. After activation via the p53/TAp63 → Notch → active Caspase 3 pathway, Caspase 3 might have a dual function. Cell autonomously caspase enhances differentiation of keratinocytes of the upper layers in breeding tubercles and simultaneously it might enhance proliferation at the base of the breeding tubercle in a paracrine way to ensure a balance between sloughing of the cap layer and proliferation at the base. This mechanism might explain how continuous renewal in tubercles is regulated.

Consequently, both isoforms of p63 would be necessary for proliferation at the base of breeding tubercles: ΔNp63 which promotes proliferation cell-autonomously as in regular zebrafish epidermis. Furthermore, TAp63 acts indirectly onto proliferation via active Caspase 3 in a non-cell autonomous way.

However, a proliferation-stimulating function of p53 might be considered as very surprising, due to its commonly known anti-proliferative function as a tumor suppressor. However newer data of metabolic functions of p53 also came to this conclusion. Loss of p53 was shown to cause severely impaired proliferation of starved human cancer cells [Maddocks et al. 2013, Maddocks et al. 2011].

It would be interesting if a similar function for TAp63 and p53 also exists in mammalian epidermis. Additionally it would shed new light onto the interactions between the different isoforms of p63.

The proposed model according to our data is depicted in **figure 41**.

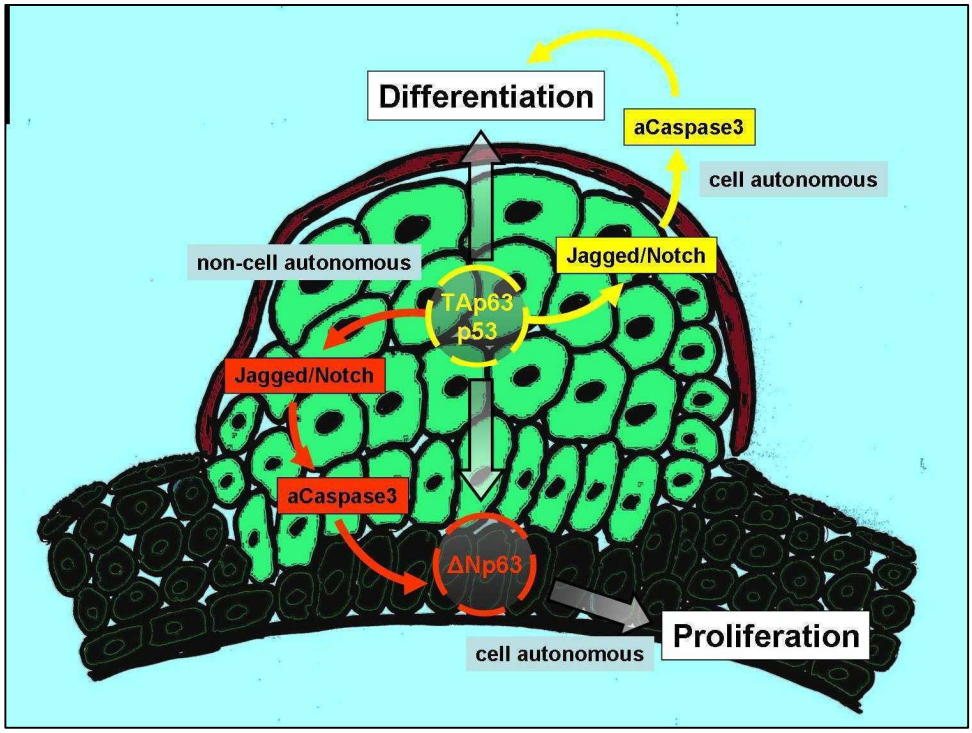

Figure 41: Proposed model of the interaction between TAp65, p53 and ΔNp63 in differentiation and proliferation of breeding tubercels of zebrafish. The interaction network involves cell-autonomous and non cell-autonomous interactions.

Redundant functions of TAp63 and p53 might at least partially explain the lack of a severe phenotype in mouse TAp63 mutants

The tubercle phenotype of the TAp63 and p53 mutants was very variable in strength and penetrance, ranging from an absolute loss of tubercles to nearly wildtype appearance. However, the same was the case for the TAp63/p53 double mutant, even if the phenotypic strength was clearly higher than in the single mutants. This leads to the suggestion that the two related transcription factor have at least partially redundant roles in the breeding tubercle formation and thus can compensate the loss of the second factor in the single mutants. However, as even in double mutants tubercle formation was not abolished completely, other factors might additionally be needed to contribute to tubercle formation. But this redundancy of TAp63 and p63 might also account for the lack of a severe epidermal phenotype in TAp63 knock-out mice [Guo et al. 2009, Su et al. 2009, Suh et al. 2006]. However, epidermis of TAp63/p53 double mutant mice has not been analyzed so far.

However, other factors might also contribute to the formation of breeding tubercles. TAp73 as the third member of the p53 family would be strong candidate, however, to our knowledge a zebrafish TAp73 specific mutant is not known so far. TAp73 was shown to be expressed in oocytes together with TAp63 to prevent genomic instability and female infertility [Dötsch et al. 2010 Tomasini et al. 2009 Tomasini et al. 2008, Suh et al. 2006].

Additionally ΔNp63 isoforms might be involved in this redundancy. In general they are thought to have dominant negative effects on TAp63 and p53 as well. Additionally they can act as transcriptional activators [Truong 2006, King & Weinberg 2003]. This contribution might additionally explain the notion that in contrast to TAp63 mutant mice, (pan-)p63 knock-out mice complemented with *krt5::ΔNp63* do display moderately impaired cornification [Candi et al. 2006].

In total, the data demonstrate the complexity of regulation, function and interplay of the different members of the p53 transcription factor family. However it seems that many of these connections are conserved during vertebrate evolution.

V. Material and Methods

1. Zebrafish methods

Zebrafish strains

Unless stated otherwise, wild-type fish from a mixture of TL and EK were used.

The mutant *Tp63* ^{hu2525} (S48X) line was generated in the Hubrecht Institute, NL, using target-selected mutagenesis (TILLING) [Wienholds et al. 2003]. The stable transgenic lines *Tg(krt4:creERt2)*^{fr33}, *Tg(krt19:dTomato)*^{fr34} and *Tg(krt19:creERt2)*^{fr35} were generated using the Tol2 kit [Kwan et al. 2007] with the described *krt4* promoter fragment [Gong et al. 2002] or a fragment of the *krtt1c19e* gene (Tom Carney; manuscript in preparation) for construct generation, followed by standard injection and screening procedures.

The mutant line *Tp53* ^{zdf1} (M214K) [Berghmans et al. 2005] and the transgenic lines *Tg(krt4:GFP)*^{gz7} [Gong et al. 2002], *Tg(actb2:loxP-STOP-loxP-dsREDEx)*^{sd5} [Bertrand et al. 2010], *Tg(TP1bglob:eGFP)*^{um13} [Parsons et al. 2009], also named *Tg(EPV.Tp1-Mmu.Hbb:eGFP)*^{um13}, *Tg(5xUAS-E1b:6xMYC-notch1a)*^{kca3} [Scheer et al. 1999], *Tg(-1.5hsp70l:Gal4)*^{kca4} [Scheer et al. 1999] and *TG(actb2:GFP)*^{zp5} [Reischauer et al. 2009] have been previously described. For NICD expression, *Tg(5xUAS-E1b:6xMYC-notch1a), Tg(-1.5hsp70l:Gal4)*^{kca4} double transgenic fish were heat-shocked from 20 – 41 dpf once a day for 1 hour at 40°C.

General fish handling

All general methods for zebrafish care and handling were performed as described in Westerfield et al. 2000. Fish rooms and incubators for embryos and larvae were kept at a constant temperature of 28°C. All tank rooms were equipped with a electronically controlled 14 hours light / 10 hours darkness cycle. Feeding was done according to the age of the fish or larvae, but at least twice a day by the animal care takers of the institute's fish facility. Staging of embryos was done according to Kimmel et al., 1995, whereas juvenile or older fish were classified according to Parichy et al. 2009.

Tamoxifen treatment

Tg(krt19:creERT2)^{fr35}*, Tg(actb2:loxP-STOP-loxP-dsREDEx)*^{sd5} double transgenic*, Tg(krt19:creERT2)*^{fr35}*, Tg(actb2:loxP-STOP-loxP-dsREDEx)*^{sd5}*, Tg(krt4:GFP)*^{gz7} triple transgenic, or *Tg(krt4:creERT2)*^{fr33}*, Tg(actb2:loxP-STOP-loxP-dsREDEx)*^{sd5}*; Tg(krt4:GFP)*^{gz7} triple transgenic embryos were treated with 5 µM 4-Hydroxytamoxifen (Sigma Aldrich; H7904) in the dark at 28°C from 24 hpf to 96 hpf before

being returned to normal system conditions for growing up. Treated fish were then imaged at regular time-points.

Fin clip and isolation of genomic DNA

Fish were anesthetized with Tricaine (ethyl-3-aminobenzoate methanesulfonate, Fluka) 1:25 in fish system water before fin clipping. The distal third of the caudal fin of adult zebrafish was cut and lysed overnight in 50 µl lysis buffer (10mM Tris pH 8.0, 2mM EDTA, 0.3% Tween20 and 0.3% glycerol) including 10 µg/ml proteinase K at 55 °C. After heat inactivation of the proteinase, the DNA was diluted 1:10 and used for genotyping.

Genotyping of TAp63 mutants

The *TAp63*^{hu2525} allele was genotyped using the dCAPS (derived Cleaved Amplified Polymorphism Sequence) method [Neff et al. 2002], with PCR primer CTG ACC CCG AGG TTG TCT AA (sense) and TGC TAA TCT GTA TAG TAT TGG AAG CT (antisense), and subsequent *HindIII* (NEB) restriction digest, yielding a smaller band for the mutant allele. The protocol was kindly provided by Krasimir Slanchev (MPI of Immunobiology, Freiburg).

TAp63MM-For2 (forward primer)	CTG ACC CCG AGG TTG TCT AA
TAp63MM-Rev (reverse primer)	TGC TAA TCT GTA TAG TAT TGG AAG CT
PCR program	1) 95°C – 2 min
	2) 95°C – 30 sec
	3) 60°C – 30 sec
	4) 72°C – 30 sec
	5) Goto 2; 40 cycles
	6) 72°C – 5 min
	7) 12°C – hold

Genotyping of p53 mutants

The *Tp53* ^{zdf1} allele was identified via an RFLP (Restriction fragment length polymorphism) genotyping assay, with PCR primer CCA GAG TAT GTG TCT GTC CA (sense) and TGA TTG TGA GGA TGG GCC TGC GGA ATC (antisense) and subsequent *Bsty*I restriction digest, yielding to a smaller band for the mutated allele.

P53-For (forward primer)	CCA GAG TAT GTG TCT GTC CA
P53-Rev (reverse primer)	TGA TTG TGA GGA TGG GCC TGC GGA ATC
PCR program	1) 95°C – 2 min
	2) 95°C – 30 sec
	3) 56°C – 30 sec

4) 72°C – 30 sec
5) Goto 2; 40 cycles
6) 72°C – 5 min
7) 12°C – hold

Genotyping of *hsp70::Gal4* and *uas::NotchICD* transgenic zebrafish

Transgene specific PCR primer were used to identify carrier fish. Primer sequences and PRC program are shown below. The expected band sizes were: hsp70::gal4: 1200 bp; uas::notchICD: 300 bp.

hsp70::gal4 transgene	
Gal4 (forward primer)	CGG GCA TTT TAC TTT TAT GTT GC
Gal4 (reverse primer)	CAT CAT TAG CGT CGG TGA G
PCR program	1) 95°C – 2 min
	2) 95°C – 30 sec
	3) 56°C – 30 sec
	4) 72°C – 100 sec
	5) Goto 2; 40 cycles
	6) 72°C – 5 min
	7) 12°C – hold

uas::notchICD transgene	
ICD (forward primer)	CAT CGC GTC TCA GCC TCA C
ICD (reverse primer)	CGG AAT CGT TTA TTG GTG TCG
PCR program	1) 95°C – 2 min
	2) 95°C – 30 sec
	3) 56°C – 30 sec
	4) 72°C – 30 sec
	5) Goto 2; 40 cycles
	6) 72°C – 5 min
	7) 12°C – hold

2. Molecular biology methods

All standard methods in molecular biology were performed according to Sambrook and Russell, 2001.

Total RNA isolation

Isolation of total RNA of embryos, larvae or adult tissue was performed with Trizole (Invitrogen) according to the recommendations of the company. Afterwards, RNA was purified by ethanol precipitation and dissolved in 50 µl RNase-free water. All RNA solutions were stored at -80°C.

RT-PCR

RNA of whole zebrafish of different developmental stages or RNA from isolated tissues/organs was isolated using the trizole reagent as described above. cDNA was amplified using random hexamer primer. 1 µg RNA was used per reaction. For the subsequent PCR reaction, the TA-specific sense primers TAS1 (CAG GGG CTA GCT TCTA GTG G), TAs2 (ATA CAT GTG CTT GGG CCA CT), TAs3 (GCA AGA CGT CCT CAA CCA GT), or the ΔN-specific sense primer ΔNs (TGT TGT ACC TGG AGA CCA ATG) were combined with a shared reverse primer (GTG ACT GGG TGG GGC TAT TT; nt501-481 of ΔNp63 CDS; NM_152986). **Figure 42** demonstrates the binding sites of the described PCR primer in the p63 sequence. Zf-bactin2 (GenBank: BC0675676) specific primers were used as positive control (Bactinfor: AGTTTGAGTCGGCGTGAAGT and Bactinrev: AGGCTGTGCTGTCCCTGTAT). The reaction was performed with an annealing temperature of 55°C for 35 cycles.

***In situ* probe synthesis**

For antisense RNA probe synthesis, a 1.8 kb fragment of *krt8* was amplified from EST GenBank BI875660 and cloned into pBluescript SK. The plasmid was linearized with *Hin*dIII (NEB) and transcribed with T3 RNA polymerase (Roche). A 0.6kb *cki* fragment was amplified from EST GenBank AF197880 and cloned into pSPORT vector. The plasmid was linearized with *Eco*RI and transcribed with SP6 RNA polymerase (Roche). A 0.4 kb *krt5* fragment was amplified from EST GenBank AF197909 and cloned into pBluescript SK vector. The plasmid was linearized with *Kpn*I (NEB) and transcribed by T3 RNA polymerase. A 1.5 kb *cke7* (ZFIN-ID now: zgc:92061) fragment was amplified from EST Genbank BI850052 and cloned into pSPORT vector. *Eco*RI (NEB) was used for linearization of the plasmid and SP6 RNA polymerase for transcription. A 1.4 kb *cyt1* fragment was amplified from EST GenBank AF084461 and cloned into pBlueskript vector. The plasmid was linearized with *EcoRI* (NEB) and T7 RNA polymerase (Roche) was used for transcription. A fragment of *krt4* was amplified from EST GenBank AF134850 and cloned into pBluescript. The plasmid was linearized with *PstI* (NEB) and T7 RNA polymerase (Roche) was used for transcription.

All other probes were cloned using cDNA specific primer (TGase1: forward primer TTACAGCCACCTGAGCACTG, reverse primer CAGGCTTTTGTCTGCAATGA). The PCR fragment was cloned into empty pCS2 vector using the TA cloning kit (Invitrogen). The plasmid was linearized with *Xho1* (NEB) followed by RNA synthesis with SP6 RNA polymerase (Roche) to obtain the antisense

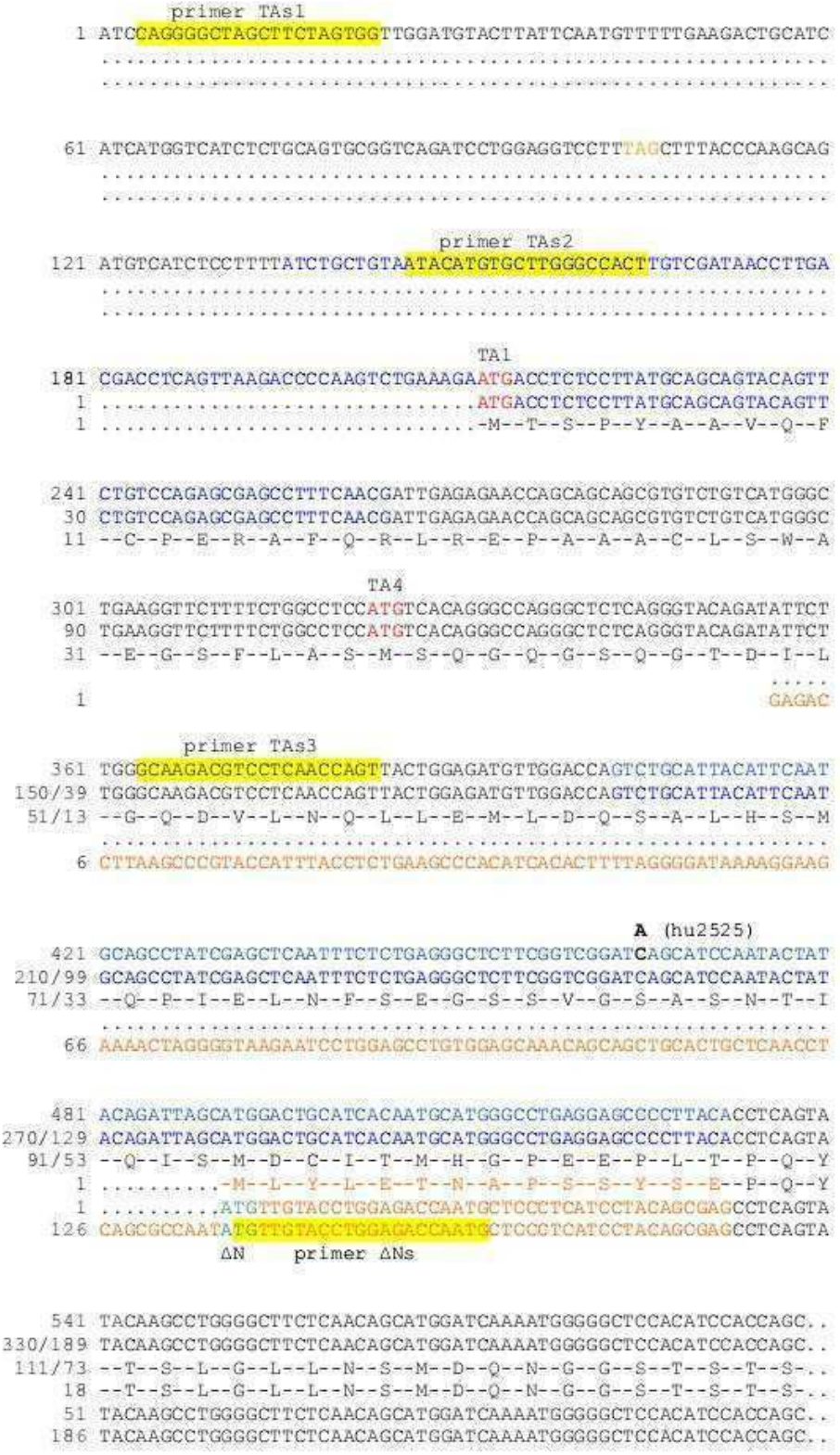

Figure 42: Binding sites of the TA and ΔN specific primer in zebrafish-p63 used for RT-PCR analysis

probe and linearized with *HindIII* (NEB) followed by RNA synthesis with T7 RNA polymerase (Roche) to obtain the antisense probe. For all probe synthesis reactions, Dig RNA labelling mix (Roche) was used as nucleotide source.

3. Histology, immunohistochemistry and microscopy

Fixation of adults and larvae

All larvae were fixed in 4% paraformaldehyde at 4°C overnight and stored in 100% methanol at -20°C. Adult fish were fixed in 4% paraformaldehyde as well, but the fixation period was carried out longer, up to three or four days.

For antibody staining with the mouse anti-pan Keratin antibody (Progen) fish were fixed with Dent's fixative (80% methanol, 20% DMSO) at room temperature for several days.

For histology staining, adult fish were fixed with Bouin's fixative (20% formaldehyde 37%, 75% picric acid, 5% acetic acid 100%) for three days at 4°C.

For transmission electron microscopy, adult tissue had to be fixed in 2% paraformadehyde + 2.5% glutaraldehyde or 2 days at 4°C. For scanning electron microscopy fish were fixed in 4% paraformaldehyde and dehydrated afterwards in 100% ethanol.

Sectioning of paraffin embedded tissue

Tissues from adult fish were embedded in paraffin for histology or antibody staining. After fixation, the tissue was decalcified with 0.5 M EDTA pH 7.4 for up to one week at room temperature on the rocker. Afterwards, the samples were dehydrated in a graded series of alcohols, cleared in Roti-Histol (Carl Roth) and embedded in paraffin wax. Finally, the paraffin blocks were sectioned using a Leica RM2255 microtome. Thickness of the sections was 8-10 µm.

Sectioning of Durcupan embedded tissue

Pecfins of adult fish and larvae were embedded in Durcupan (Fluka) embedding medium directly after the fixation. The tissue was dehydrated in an ascending ethanol series and finally in acetone. The tissue was incubated overnight in a 1:1 acetone/Durcupan solution mixture. On the next day, tissue samples were transferred to embedding moulds and orientated in fresh Durcupan solution. The Ducrupan polymerized while incubation at 65°C overnight.

For all experiments Durcupan medium solidity was used: 32 ml solution A mixed with 27 ml solution B, 1 ml solution C and 0.6 ml solution D.

Durcupan blocks were cut with the same microtome as paraffin sections, but specific disposable high profile for hard tissue were used.

Cryo-sections of cryo-embedded tissue

After fixation the tissue was soaked in 30 % sucrose in PBST at 4 °C overnight. On the next day, the tissue blocks were orientated in melted 1.5% agarose in 15 % sucrose in PBST. After the gel had polymerized the tissue samples were cut according to the required orientation and stored until use in 30% sucrose in PBST. At the cryostat, an embedding plate was prepared with freezing medium in -80 °C cold isopentene. The agarose block with the tissue was mounted onto the plate and covered with freezing medium and shock-frozen in isopentene. The cryostat Leica CM1850 was used to perform 10-14 µm thick cryo-sections. The sections could be stored at 4 °C until further usage.
Cryo sections were performed especially from transgenic lines, when the endogenous fluorophore of the transgene should be visualized without previous immunohistochemistry.

Whole mount *in situ* hybridization on larval zebrafish

The in situ hybridization protocol was performed according to [Nüsslein-Volhard & Dahm 2002] with some modifications. Zebrafish embryos / larvae were stored in 100% methanol at -20°C and rehydrated before the start. The fish were digested with 10µg/ml proteinase K at room temperature according to the embryonic stage (2 dpf 45 min, 3dpf 60 min). Afterwards the embryos were refixed in 4% paraformaldehyde for 20 min at room temperature. After washings, the embryos were prehybridized in prehybridized in a freshly prepared hybridization solution (Hyb+) for several hours at 65°C. The hyb+ was exchanged by the digoxygenin-labelled probe (diluted in hyb+ 1:100) and incubated overnight at 65°C. . On the next day the samples were washed at 65°C with 75% Hyb -/ 25% 2xSSCT, 50% Hyb-/ 50% 2xSSCT, 25% Hyb-/ 75% 2xSSCT and 2xSSCT for 15 min each. Then they were washed twice with 0.2xSSCT for 30 min at 65°C, followed by washings with PBST at room temperature. In the following, the embryos were blocked in blocking solution at room temperature over several hours. The blocking solution was replaced by anti-DIG Fab fragment dilution (1:500 in blocking solution) and the embryos incubated at 4°C overnight. The anti-DIG antibody was coupled with alkaline phosphatase that was used later for the staining reaction. On the next day, the samples were washed extensively and incubated three times in staining solution for 5 min at room temperature. The staining was performed with NBT/BCIP (Roche) (dilution 1:20 in staining buffer) at room temperature in the dark. The staining reaction was stopped by washing in PBST.

Special solutions / reagents:	Staining buffer 0,1M Tris pH9,5; 50mM MgCl; 0,1M NaCl; 0,1% Tween20 NBT/BCIP (Roche) Hyb - 50% formamid, 5xSSC, 0,1%Tween20; 9,2mM citric acid

Hyp+
Hyp- enriched with 50µg/ml Heparin and 0,5mg/ml tRNA

In-situ hybridization of paraffin sections

The protocol was performed according to Moorman et al., 2001 with slight modifications.
The sections were rehydrated in a descending Roti-histol/ethanol series and finally washed in PBST, followed by permeabilization with proteinase K solution 10 µg/ml in PBS at 37°C for 10 min. The digestion was stopped with 0.2% glycine solution in PBS and the sections briefly re-fixed with 4% paraformaldehyde with 0.2% glutaraldehyde for 20 min at room temperature. After washing, the sections were pre-hybridizided for several hours at 65°C in hybridization mix. The RNA antisense probe was heated up to 80°C for 10 min to destroy secondary structures. The hybridization mix was replaced by 80 µl diluted RNA antisense probe (all probes were diluted 1:100 in hybridization mix, the slide covered with a coverslip and incubated overnight at 65°C.
On the next day, stringency washings were performed with 50% formamide + 2x SSC at 65°C, twice for 20 min. After several washings in PBST, unspecific binding sites were blocked by incubation with 100 µl blocking solution per slide in a humidified box for several hours at room temperature. Subsequently, the blocking solution was replaced by anti-DIG-FAB solution 1:5000 (in blocking solution). For ech slide, 100 µl antibody solution were used. The slides were covered by cover slips and incubated over night at 4 °C. After extensive washing, the sections were incubated three times in staining buffer for 5 min, before the staining reaction was performed with NBT/CIP according to the manufacturer's recommendations. During staining, the slides were kept in the dark. The staining reaction was stopped by washing with PBS. Finally, the slides were mounted with Mowiol.

For hybidizazion mix, anti-DIG Fab (Roche) solution and the NBT/BCIP staining reaction, the same reagents were used as described in the previous section.

Immunostaining

Paraffin sections were rehydrated in a descending Roti-histol/ethanol series. For antigen retrieval, the slides were heated up to 70°C in 0.1 mM citrate buffer pH 6.0 for 2 hours. After the slides returned to room temperature, they were washed with PBST and incubated several hours in blocking solution. Incubation with the primary antibody diluted in blocking solution was performed overnight at 4°C. On the next day, sections were washed thoroughly with PBST, blocked again with blocking solution and incubated with the secondary antibodies at 4°C overnight. After final washings in PBST, the slides were mounted with Mowiol/DAPI.
For double staining, the sections were incubated with both primary and both secondary antibodies respectively, at the same time.

Blocking solution: 10% fetal calf serum with 0.1% DMSO inPSBT.

Primary antibodies used in the described experiments:

Antibody	**Company**	**Organism**	**dilution**
anti-panKrt(1-8) TypeII	Progen 61006	mouse	1:200
anti-p63 (4A4)	Santa Cruz sc-8431	mouse	1:150
anti-ZO1	Zymed 33-9100	mouse	1:200
anti-BrdU	Roche 1170376	mouse	1:200
Anti-active Caspase 3	abcam ab-13847	rabbit	1:1000

Secondary antibodies used were: anti-mouse Cy3 (1:1000, Invitrogen), anti-rabbit Cy3 (1:1000, Invitrogen), AlexaFluor-488 anti-rabbit (1:1000, Invitrogen)

Hematoxylin / Eosin (HE) staining:

Paraffin sections were rehydrated with washings in Roti-histol and decreasing ethanol series as described above. The slides were bathed in hematoxylin (Merck) solution for 30 sec followed by 5 min of washing under running tab water. Bathing in eosin solution (Merck) was done for 20 min, again followed by washing out under running water. Finally, the slides were again dehydrated with the ethanol/Roti-Histol series in reverse direction and mounted with Entellan.

Masson-Goldner-Trichrome (MGT) staining

MGT staining was performed with the Masson-Goldner Staining kit by Merck according to the manufacturer's recommendations. Slides were mounted with Entellan after the final dehydration series.

Periodic acid shift (PAS) staining

After rehydration, sections were oxidized with 0.5% periodic acid solution for 5min. Afterwards the sections were bathed in Schiff's solution (Merck) for 15 min. After washing with tap water, nuclei were counterstained with Weigert's hematoxylin solution for 5 min. After washing, slides were mounted with Entellan.

TUNEL (TdT mediated dUTP nick end labelling) assay on sections

TUNEL assay on sections was performed using the In Situ Cell Death Detection Kit, POD (Roche) according to the manufacturer's instructions.

The sections were rehydrated in a Roti-histol/ethanol series with decreasing amount of ethanol as described above. Antigen retrieval was performed by incubation in 0.1 M citrate buffer pH 6.0 at 70°C for two hours. After washings with PBST, the TUNEL reaction was performed according to the

manufacturer's protocol. A reaction mix without the TdT enzyme was used as negative control, whereas digestion of sections with Dnase I prior to the TUNEL reaction was the positive control. After washing, signal conversion was done with the kit POD enzyme according to the company's protocol. Finally, the staining reaction was performed with DAB substrate for 10 min.

Acridine orange staining

Acridine orange staining was performed as described in Furutani-Seiki et al. 1996 with slight modifications. Adult zebrafish were bathed in 5 mg/ml acridine orange (Sigma) in fish water for 30 min at 28°C in the dark followed by extensive washings. The fish were immediately anaesthetised with Tricaine 0.1% and observed under the fluorescence stereomicroscope using the GFP filter.

Methylene blue assay

After anaesthetisation, adult fish were stained in 100 mg/ml methylene blue solution for 1 min, rinsed with clear water and observed immediately under the binocular.

Calcein / Calcein blue staining

Adult or juvenile fish were bathed in 100 mg/l calcein (Sigma) or calcein blue (Sigma) solution at room temperature for 2 hours in the dark. After quick washing steps, the fish were anaesthetized with Tricaine 0.1% and observed with the stereo microscope using the GFP filter.

BrdU staining

The fish were stained in 100 µg/ml BrdU (Sigma) solution for 24 hours at 28 °C in the dark. The fish were washed several times with fish water and scarified after further four hours at 28 °C. Detection of BrdU was performed as antibody staining as described above using an anti-BrdU primary antibody. The only modification to the regular antibody staining protocol was an additional incubation step with 1 N HCl at room temperature for 1 hour directly after the antigen retrieval step.

Chemical inhibitor treatment

Fish were raised from 20 dpf – 50 dpf in E3-medium containing 100 µg/ml of the γ-secretase inhibitor DAPT (N-N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycien t-butylester; Sigma-Aldrich 208255) [Geling et al. 2002], 5 µg/ml of the caspase 3 peptide inhibitor z-DEVD-fmk (Calbiochem 264155-80) [Parng et al. 2004] or 0.2% DMSO as control. To allow feeding, the fish were transferred to a 1L tank for 5 hours a day and afterwards returned into the inhibitor solution. The same was done with the

DMSO treated control group. The inhibitor solutions were renewed twice a week. The whole experiment was performed under standardized conditions at 28 °C.
Standard length (SL) of the larvae was used to control equal development of each treatment group.

Fluorescence and bright light microscopy

All immunohistochemistry was done with fluorescent secondary antibodies. The sections were mounted with Mowiol containing 10 µl DAPI. Microscopy was done with a Zeiss Axioimager Z1 (containing the Apotome module) or M1.
In situ hybridization sections and TUNELs were evaluated with an Axiophot microscope (Zeiss), after the slides were mounted with Entelan.
For stereomicroscopy, living fish were anaesthetized with 0.1 % tricaine (Sigma) and evaluated with a fluorescent Leica M165 FC.

Preparation for Electron microscopy (EM)

All samples for transmission EM (TEM) were fixed in 2 % paraformaldehyde + 2.5 % glutaraldehyde in PBS at 4°C over night. The samples were not decalcified. All sectioning was done by Mojgan Ghilav (Deutsche Sporthochschule, Cologne). Imaging was done with the help of Prof. Wilhelm Bloch (Deutsche Sporthochschule, Cologne).
All scanning EM (SEM) samples were fixed in 4 % paraformaldyhyde at 4°C overnight. Either the whole adult fish was fixed or the head was separated from the trunk prior to fixation. All samples were dehydrated via ethanol series with increasing amount of ethanol. Microscopy was performed at the Max Planck Institute for Plant Breeding Research in collaboration with Dr. Elmon Schmelzer and Rainer Franzen. Dehydrated samples were either cryo-fixed, sputter-coated (gold/palladium) and evaluated in frozen stage, or critical point dried (CPD), sputter-coated and evaluated under room temperature conditions. Initial samples were mounted by Thomar Ramezani.

VI. Abbreviations

AO	Acridine orange
CE	Cornified envelope
CPD	Critical point drying
DBD	DAN binding domain
dpf	Days post fertilization
ECM	Extracellular matrix
EVL	Enveloping layer
GFP	Green fluorescence protein
HE	Hematoxylin / Eosin
IFAP	Intermediate filament associated protein
K	Keratin
KHLG	Keratohyalin-like granula
KIF	Keratin intermediate filaments
mGFP	Membrane bound GFP
MGT	Masson-Goldner trichrome
mRNA	Messenger RNA
NICD	Notch inracellular domain
OD	Oligomerization domain
PAS	Periodic acid shift
PFA	Paraformaldehyde
RBP-J	Recombination signal binding protein J
RE	Responsive element
SEM	Scanning electron microscopy
SL	Standard length
SPRP	Small prolin rich protein
TAD	Transactivation domain
TEM	Transmission electron microscopy
TGM	Transglutaminase
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling

VII. Literature

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Erklärung

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