Česká zemědělská univerzita v Praze

Fakulta agrobiologie, potravinových a přírodních zdrojů

Katedra veterinárních disciplín

Charakterizace procesů nezbytných pro zahájení embryonální genomové aktivace u preimplantačních embryí savců

doktorská disertační práce

Autor práce: Ing. Veronika Kinterová

Školitel: doc. Ing. Eva Chmelíková, Ph.D.

Konzultant: RNDr. Jiří Kaňka, DrSc.

Čestné prohlášení

Prohlašuji, že svou disertační práci „Charakterizace procesů nezbytných pro zahájení embryonální genomové aktivace u preimplantačních embryí savců" jsem vypracovala samostatně pod vedením vedoucího práce a s použitím odborné literatury a dalších informačních zdrojů, které jsou citovány v práci a uvedeny v seznamu literatury na konci práce. Jako autorka uvedené disertační práce dále prohlašuji, že jsem v souvislosti s jejím vytvořením neporušil autorská práva třetích osob.

V Praze dne 20.09.2019 _____

Poděkování

Ráda bych touto cestou poděkovala doc. Ing. Evě Chmelíkové, Ph.D. za vedení mého doktorského studia a odborné konzultace. Dále bych chtěla poděkovat RNDr. Jiřímu Kaňkovi, DrSc. za jeho vstřícný přístup, rady a připomínky, které mi během studia i výzkumu poskytl. Ráda bych dále poděkovala i ostatním členům Laboratoře vývojové biologie za vytvoření přátelského prostředí a předání cenných zkušeností. V neposlední řadě mé poděkování patří rodině za podporu během celého studia.

Charakterizace procesů nezbytných pro zahájení embryonální genomové aktivace u preimplantačních embryí savců

Abstrakt

Časný preimplantační vývoj savců je řízen maternálními mRNA a proteiny, které byly nasyntetizovány již v průběhu oogeneze. Během časného vývoje jsou tyto rezervy postupně odstraňovány a nahrazeny jejich embryonálními formami. Zatímco degradace maternálních mRNA je postupný proces, který vrcholí v období okolo hlavní vlny aktivace embryonálního genomu (EGA), o degradaci maternálních proteinů není známo příliš mnoho informací. Jedním z nejpravděpodobnějších způsobů odstranění maternálních proteinů se jeví degradace pomocí ubiquitin – proteazomového systému (UPS). K tomu, aby byly proteiny odstraněny pomocí proteazomu, musí být nejprve označeny ubiquitinem (tzv. ubiquitinace). Na ubiquitinaci se podílí tři enzymové komplexy: E1, E2 a E3 enzymy. Výběr specifického cílového proteinu a finální navázání ubiquitinu je zprostředkováno pomocí E3 – ubiquitin ligáz.

Tato práce se soustředila na detailní studium SCF komplexu (Skp1-Cul1-F-box), jedné z nejčastějších E3 ubiquitin-ligáz. SCF komplex se skládá ze tří neměnných složek: Cul1, Skp1, Rbx1 a jednoho zástupce z rodiny F-box proteinů, který určuje substrátovou specifitu. Nejprve jsme sledovali expresi mRNA a proteinů jednotlivých neměnných členů SCF komplexu. Počátek syntézy mRNA Cul1 a Skp1 byl detekován u embrya skotu již v 4buněčném a časném 8buněčném stádiu, což naznačuje nezbytnost těchto transkriptů pro přípravu embrya na EGA. Hladina proteinu CUL1 postupně vzrůstala od MII oocytů po stádium moruly a protein byl lokalizován především v jádrech, méně v cytoplazmě. Ve stádiu blastocysty byla sledována nižší intenzita signálu ve vnitřní buněčné mase (ICM) než v trofoektordermu (TE). Hladina proteinu SKP1 signifikantně vzrostla mezi MII a 4buněčným stádiem, následně však opět signifikantně poklesla. Tento protein byl lokalizován rovnoměrně rozprostřený v cytoplazmě, ve stádiu blastocysty s mírně nižší intenzitou v oblasti ICM. V hladinách proteinu RBX1 nebyl detekován žádný statisticky významný rozdíl, i jeho lokalizace v cytoplazmě byla po celý vývoj nezměněná. Aktivní SCF komplex, který je určen vazbou Cul1 na Skp1, byl rovnoměrně rozložen v průběhu celého preimplantačního vývoje. Ve stádiu blastocysty však byla jeho aktivita znatelně nižší v oblasti ICM než v oblasti TE.

K inhibici SCF ligáz jsme použili MLN4924, což je inhibitor neddylací aktivovaných ligáz. Po kultivaci oocytů v tomto inhibitoru nedocházelo k expanzi kumulárních buněk, nicméně oocyty byly úspěšně *in vitro* oplozeny. Po oplození jsme však detekovali signifikantní nárůst polyspermie a i následující vývoj do stádia blastocysty byl zhoršený. Opožděný vývoj jsme nalezli i při využití inhibitoru MLN4924 ke kultivaci embryí od 4buněčného do pozdního 8buněčného stádia. U těchto embryí jsme detekovali snížené množství mRNA markerů EGA – PAPOLA a U2AF1, což naznačuje opožděnou aktivaci embryonálního genomu. Kultivace v MLN4924 vedla ke zvýšení celkového množství proteinů u ošetřených oocytů i embryí, u kumulárních buněk se podobný nárůst neprojevil. Pomocí metody western blot jsme sledovali hladinu následujících proteinů: SMAD4, ribozomální protein S6, centromerický protein E, P27, IκBA, RBM19 a ZAR1. Neobjevili jsme však žádný statisticky významný nárůst v jejich množství, který by značil jejich hromadění. U třech proteinů (P27, IκBA, SKP1) jsme při western blot analýze všech vývojových stádií nalezli bandy s vyšší molekulární hmotností, než bylo očekáváno. Tyto vyšší bandy pravděpodobně naznačují tvorbu komplexů, které mohou být pro preimplantační embrya specifické.

Naše práce prokázala, že SCF ligázy jsou nepostradatelné pro správné zrání oocytů, expanzi kumulárních buněk, oplození a časný preimplantační vývoj skotu.

Klíčová slova: preimplantační embrya, aktivace embryonálního genomu, degradace proteinů, ubiquitin – proteazomový systém, SCF komplex

Characterization of processes necessary for the initiation of embryonic genome activation in mammalian preimplantation embryos

Abstract

The early preimplantation development is controlled by maternal mRNA and proteins synthetized during oogenesis. These reserves are gradually degraded and replaced by embryonic transcripts during early embryogenesis. The degradation of maternal mRNA is gradual process, which peaks around the major wave of embryonic genome activation (EGA), but mechanisms underlying the elimination of maternal proteins remains still unknown. One of the most likely way to eliminate maternal proteins seems to be ubiquitin – proteasome system (UPS). UPS-based protein degradation is managed by ubiquitin attached to the targeted protein (process called ubiquitination) by the cooperation of three enzymatic complexes: E1, E2 and E3 enzymes. E3 enzymes are responsible for protein specificity and final binding of ubiquitin to the target protein.

These thesis is focused mainly on a detailed study of SCF (Skp1-Cul1-F-box) complex, one of the most common E3 ubiquitin-ligases. The SCF complex is composed by three invariant members: Cul1, Skp1, Rbx1 and one member from the F-box family, which determines the substrate specificity. At first, we observed the expression of mRNA and proteins of all three invariable members. Cul1 and Skp1 mRNA synthesis was detected at the four-cell and early eight-cell stage respectively, which suggests that these transcripts are necessary for preparing the embryo for EGA. CUL1 protein level increased gradually from MII oocytes to the morula stage embryos and protein was localized primarily to nuclei and to a lesser extent to the cytoplasm with a lower signal in the inner cell mass (ICM) compared to trofoectoderm (TE) at the blastocyst stage. The level of the SKP1 protein significantly increased from MII oocytes to four-cell stage embryos, but then significantly decreased again. The localization of the SKP1 protein was analysed throughout the cell and staining was less intensive in the ICM at the blastocyst stage. There were no statistical differences in RBX1 protein level and this protein was localized in cytoplasm during all preimplantation development without changes. The active SCF complex, which is determined by the interaction of Cul1 and Skp1, was found throughout the whole embryo during preimplantation development. The signal of active complex was less intense in the ICM than in the TE.

After the SCF ligases inhibition by cultivation in MLN4924, inhibitor of neddylation controlled ligases, we found no cumulus cells expansion, however oocytes were able to be *in vitro* fertilized. After fertilization we detected significantly higher rate of polyspermy and deteriorated embryonic development. After treatment with MLN4924 from the four-cell to late eight-cell stage, we found a statistically significant delay in their development. We found reduced levels of mRNA of EGA markers – PAPOLA and U2AF1, which indicated delay in embryonic genome activation. The cultivation with MLN4924 caused a significant increase in protein levels in MLN4924-treated oocytes and embryos, no such change was found in cumulus cells. To detect, which proteins are affected by MLN4924 treatment, we performed western blot analysis of selected proteins: SMAD4, ribosomal protein S6, centromeric protein E, P27, IκBA, RBM19 and ZAR1. No statistically significant increase in protein levels was detected in either treated oocytes or embryos. However, we found bands of higher molecular weight than expected in three proteins (P27, IκBA and SKP1) after western blot analysis of all developmental stages. These higher bands can indicate complexing of proteins specific for preimplantation embryos.

Our study showed that SCF ligases are necessary for the correct maturation of oocytes, cumulus cells expansion, fertilization, and early preimplantation development of cattle.

Key words: preimplantation embryos, embryonic genome activation, protein degradation, ubiquitin – proteasome system, SCF complex

Obsah

1. Úvod

V posledních letech zažívá odvětví biotechnologie a *in vitro* oplození (IVF) prudký rozvoj. Nejnovější poznatky jsou využívány nejen v zemědělství, ale také ve zdravotnictví v oblasti asistované reprodukce. Porozumění mechanizmům a událostem probíhajícím v časném embryonálním vývoji je pro další pokrok stěžejní. V průběhu preimplantačního vývoje savců se setkáváme s několika kritickými ději, které jsou pro další vývoj klíčové. Mezi tyto procesy patří především aktivace embryonálního genomu a následná diferenciace embrya. Preimplantační vývoj člověka a skotu se do značné míry podobají, proto jakýkoliv nový poznatek týkající se bovinního embryonálního vývoje může sloužit ke zdokonalení nejen zemědělské produkce, ale také humánní asistované reprodukce.

Během oplození musí dojít ke spojení dvou vysoce specializovaných gamet, které vytvářejí kompletně nový organizmus. V počátečním úseku vývoje je genom transkripčně inaktivní, aby umožnil přeprogramování zygoty do totipotentního stavu. Postupně dochází k aktivaci embryonálního genomu, vývoj začíná být kontrolován embryem a maternální zásoby jsou nahrazovány embryonálními. I přes desítky let aktivního výzkumu embryonálního vývoje řady modelových organizmů, chybí úplné porozumění všech mechanizmů, které řídí přechod k embryonální kontrole vývoje a úspěšnou aktivaci embryonálního genomu.

Časný embryonální vývoj je řízen maternálními mRNA a proteiny. Ty jsou syntetizovány v průběhu oogeneze a následně využívány v počátečních stádiích embryogeneze. Během následujícího vývoje jsou maternální rezervy postupně nahrazovány embryonálními. Degradace maternální mRNA je postupný proces, který vrcholí v období okolo hlavní vlny aktivace embryonálního genomu (EGA). Způsob, kterým jsou z embrya odstraněny maternální proteiny, však doposud nebyl objasněn. Odstranění maternálních proteinů je pravděpodobně pro následující vývoj důležité, podobně jako odstranění maternální mRNA. Jejich degradace ale patrně nebude tak rychlá a některé proteiny by mohly být zachovány i po EGA. Jedním z nejpravděpodobnějších způsobů odstranění maternálních proteinů se jeví degradace pomocí ubiquitin – proteazomového systému (UPS). Prozatím však není příliš jasné, které enzymy tohoto systému se na jejich degradaci podílejí, a proto je v tomto směru nutný ještě další výzkum.

2. Literární přehled

2.1. Oplození

Embryonální vývoj všech savců začíná splynutím haploidní spermie s haploidním oocytem za vzniku diploidní jednobuněčné zygoty. Kontaktem se spermií oocyt znovuobnoví meiózu zastavenou v metafázi II (MII) druhého meiotického dělení a vstoupí do interfáze. Spermie poskytne svou DNA k tvorbě samčího prvojádra. Ostatní součásti spermie, jako jsou mitochondrie, prekurzory mikrotubuly-organizujícího centra a další buněčné komponenty, pravděpodobně nehrají v oplození a dalším embryonálním vývoji žádnou významnou roli (Saunders et al. 2002). Oocyt je zodpovědný za rozpoznání spermie, prevenci polyspermie, remodelaci paternálního genomu, aktivaci embryonálního genomu a samotný přechod z maternální na embryonální kontrolu vývoje (Li et al. 2013). Po dokončení meiózy a vzniku 1buněčného embrya, tzv. zygoty, dojde k replikaci paternální i maternální DNA a první buněčné dělení je pozorováno přibližně 24 hodin po oplození (Laurincík et al. 1998).

Spermie během průchodu reprodukční soustavou samice podstupují metabolické a enzymatické změny a změny ve schopnosti vázat se na vrstvu *zona pellucida* (ZP) (Mújica et al. 2003). Tyto procesy se nazývají kapacitace a akrozomální reakce a u skotu trvají přibližně 6 hodin. Pod kapacitaci spadají především enzymatické a strukturální modifikace akrozomu a přední části hlavičky spermie. Patří sem zvýšení propustnosti membrány pro vápník, modifikace struktury membrány a přeměna proakrozinu na akrozin. Kapacitace se stimuluje při vstupu spermie do reprodukčního traktu. Akrozomová reakce zahrnuje fúzi membrány spermie a akrozomu a formaci otvorů, kterými se mohou uvolňovat enzymy akrozomu a následně rozrušit mezibuněčné spoje buněk obklopující oocyt. Díky tomu se spermie dostane do přímého kontaktu s membránou vajíčka a může dojít k samotnému splynutí membrán (Ball & Peters 2008).

Po kontaktu spermie dojde v oocytu k vypuštění intracelulárních Ca^{2+} iontů, které oscilují v cytoplazmě oocytu (Saunders et al. 2002). Tato oscilace spustí řadu změn souhrnně označovaných jako aktivace oocytu. Mezi tyto procesy patří exocytóza kortikálních granul, polyspermický blok tvořený vrstvou *zona pellucida* a znovuobnovení buněčného cyklu (konec druhého meiotického bloku) (Kline & Kline 1992). Základem oscilace vápenatých kationtů je uvolňování z intracelulárních zásob, které zprostředkovává inozitoltrisfosfát (IP3). Ten vzniká hydrolýzou z fosfatidylinositol-4,5-bisfosfátu (PIP2) pomocí PLCζ, konkrétního druhu fosfolipázy C, která je specifická pro spermie. PLCζ difunduje do vajíčka po splynutí membrán

spermie a oocytu (Schultz 2005). Dále nastává blok polyspermie, což je několikastupňový proces, který je primárně realizován na dvou úrovních – pomocí plasmatické membrány oocytu a vrstvou *zona pellucida*. Na plazmatické membráně oocytu se vyskytuje receptor Juno, který je schopný se vázat na samčí protein IzumoI vyskytující se na povrchu kapacitované spermie. Při oplození dojde k jejich vzájemné vazbě a následnému odstranění receptorů Juno z povrchu oocytu. Díky tomu je zabráněno vazbě dalších spermií, potažmo vzniku polyspermie (Bianchi et al. 2014). Dalším způsobem zamezení polyspermie je pomocí vrstvy *zona pellucida*. *ZP* je mimo blokování polyspermie důležitá i pro rozeznání druhově specifických spermií, brání rozptylu blastomer, ochraňuje embryo během časného vývoje a usnadňuje jeho průchod vaječníkem (Vanroose et al. 2000). Obecně se ZP skládá pouze z několika vysoce-modifikovaných glykoproteinů: ZP1, ZP2, ZP3 a ZP4, jejichž zastoupení se u jednotlivých druhů může lišit. U skotu nebo člověka jsou zastoupeny všechny z uvedených proteinů, ale například u myši chybí ZP4, protože v jeho genu se vyskytuje několik stop kodonů (Lefièvre et al. 2004). U skotu tedy nalezneme ZP1, ZP2, ZP3-α, ZP3-β a ZP4 (Topper et al. 1997). Každý z těchto glykoproteinů plní jinou funkci. ZP3-α je receptorem spermie, ZP2 slouží jako sekundární receptor spermií reagující s akrozomem. Po fúzi spermie a oocytu dochází k modifikacím ZP2 a ZP3 proteinů v následku exocytózy kortikálních granulí oocytu, díky kterým blokují tyto glykoproteiny polyspermii (Vanroose et al. 2000; Li et al. 2013). Polyspermie je pro časná embrya letální (Li et al. 2013).

2.2. Preimplantační vývoj

Časný embryonální vývoj je velice složitý a striktně regulovaný proces, během kterého se diferenciovaný oocyt přemění na totipotentní blastomery, které tvoří embryo v počátcích vývoje. Krátce po průniku spermie do oocytu dochází k obnovení meiózy a formuje se samčí a samičí prvojádro (Barnes & Eyestone 1990). První buněčné cykly po oplození jsou rychlé z důvodu chybějících nebo velmi krátkých G fází. Jednotlivé blastomery nerostou, syntéza proteinů je omezená a embryo využívá převážně zásoby nasyntetizované a uložené již v průběhu oogeneze. Na rozdíl od nižších obratlovců je však počáteční dělení savčího embrya pomalejší. Zatímco *Xenopus laevis* či *Drosophila melanogaster* jsou schopni prodělat několik prvních dělení během 3 hodin po oplození (hpf), první dělení savců se objevuje zhruba 18-36 hpf a následující dělení přibližně každých 12 – 24 hodin až do stádia blastocysty (Jukam et al. 2017). U savců můžeme pozorovat asynchronní dělení, kdy je možné sledovat v jednu chvíli lichý počet blastomer (Gilbert 2000).

Ve stádiu 32 buněk začíná u embryí skotu kompaktace a vzniká morula (Van Soom et al. 1997). Kompaktace je první morfologický proces vývoje a, spolu s následnou kavitací, je řízena expresí faktorů, které ovládají adhezi buněk a diferenciaci trofoektodermu (Watson & Barcroft 2001). Během kompaktace se mezibuněčné kontakty mezi blastomerami zvyšují, dokud obrysy jednotlivých blastomer nezmizí. Diferenciace trofoektodermu začíná spolu s počátkem polarizace buněk vnějších blastomer během kompaktace (Watson & Barcroft 2001). V tomto období dochází k první specializaci některých buněk a začíná se tvořit vnitřní buněčná masa (ICM) a trofoektoderm (TE), ze kterých se následně vytvoří embryonální a extraembryonální tkáň. Pro adhezi buněk je nejdůležitější E-kadherin. Embrya s inhibovaným E-kadherinem sice kompaktují, ale nevyvinou se z nich blastocysty a následně nezvládají opustit *ZP* (tzv. hatchovat) (Watson & Barcroft 2001). Komunikace mezi jednotlivými buňkami je zajištěna díky mezerovým spojům (gap junctions). Jejich přítomnost se však u *in vitro* a *in vivo* embryí liší. Zatímco v ICM *in vivo* i *in vitro* embryí můžeme gap junctions nalézt, v trofoektodermu *in vivo* embryí můžeme tyto spoje nalézt pouze v omezené míře, u *in vitro* embryí gap junctions v oblasti trofoektodermu dokonce úplně chybí. Tyto rozdíly mezi jednotlivými oblastmi embryí mohou být dány odlišnými potřebami v komunikaci. Zatímco blastomery ICM potřebují intercelulární komunikaci pro koordinaci formování embrya, blastomery trofoektodermu slouží především jako epiteliální bariéra, a proto komunikace mezi nimi nemusí být tak potřebná (Boni et al. 1999).

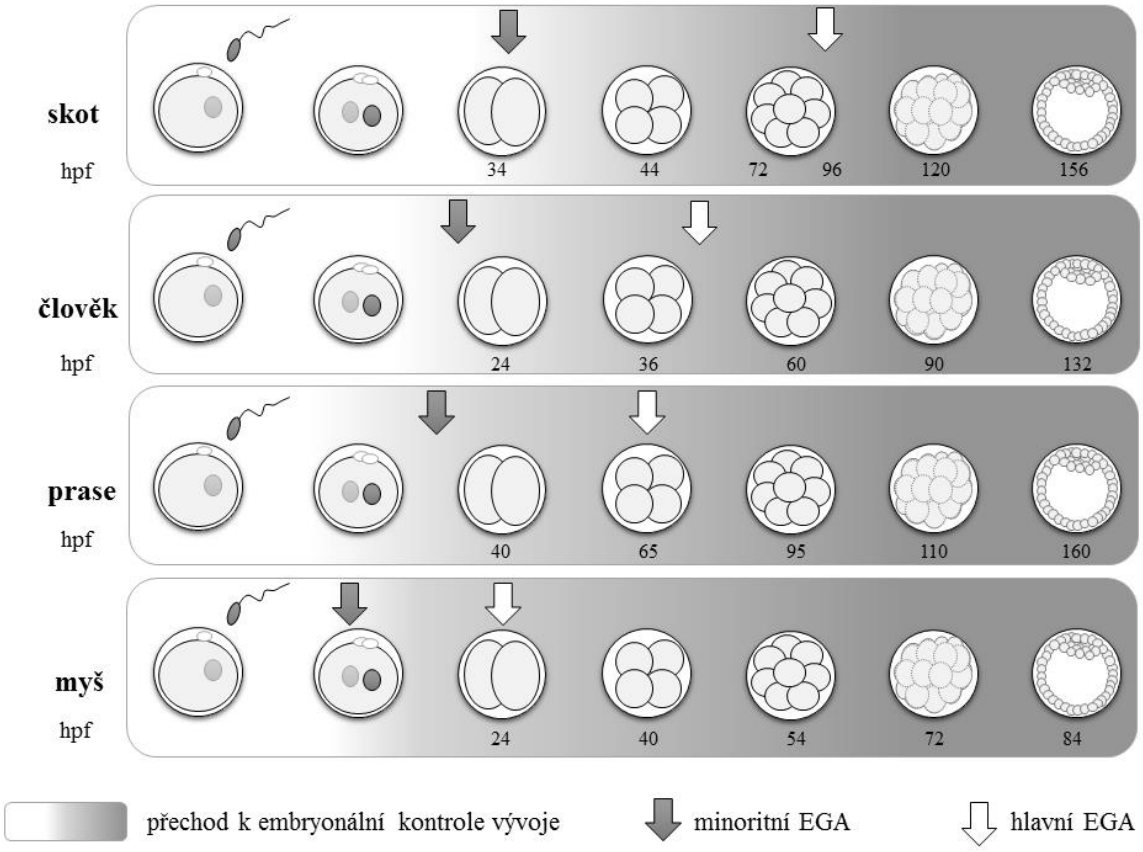
Tvorba blastocoelu nezačne dříve, než embryo dosáhne 64buněčného stádia. *In vitro* blastocysty vznikají však dříve, během 6. buněčného dělení, tzn. mezi 32 a 64buněčným stádiem (Van Soom et al. 1997). Tvorba blastocysty je řízena expresí specifických genů (Watson & Barcroft 2001). Pro diferenciaci trofoektodermu jsou důležité faktory Eomes, Tead4 a Cdx2. Naopak pro udržení pluripotence buněk ICM je nutný Oct4, Nanog a Sox2. Objevují se zde však mezidruhové rozdíly, kdy například Oct4 můžeme pozorovat kromě ICM také u blastomer TE u skotu, prasete nebo člověka (Berg et al. 2011). Buňky trofoektodermu se zplošťují a vytváří mezi sebou těsné spoje (tight junctions) (Ducibella & Anderson 1975). Buňky TE pumpují pomocí Na/K ATPázy do intracelulárních mezer tekutinu bohatou na sodíkové ionty. Přenos iontů pomocí buněk TE hraje důležitou roli pro udržení koncentračního gradientu a vytváří tak hnací sílu pro prostup vody pomocí akvaporinů. Voda je nasávána do středu embrya a vzniká blastocoel (Bowman & McLaren 1970). Se zvyšujícím se množstvím tekutiny uvnitř blastocysty dochází k jejímu zvětšování. Jak blastocysta expanduje, *zona pellucida* se ztenčuje a embryo se postupně přesouvá do dělohy. Embryo skotu vstupuje do

dělohy přibližně 4. – 5. den po oplození (Ball & Peters 2008; Valadão et al. 2018). V děloze dojde k opuštění *ZP* a embryo je připravené k implantaci do děložní sliznice, aby mohlo přijímat výživu životně důležitou k jeho následujícímu, postimaplantačnímu vývoji (Jukam et al. 2017). Embryo skotu se uhnízdí (tzv. implantuje) do dělohy až přibližně 30. den po oplození, zatímco embryo člověka již přibližně 9. den a myši již 4. den po oplození (Lee & DeMayo 2004). U embryí skotu se setkáváme s tzv. elongací blastocysty, tedy s jejím prodlužováním a přechod z vejcovitého na vláknitý tvar. Dochází k rychlému vývoji trofoektodermu, který tak poskytuje větší plochu pro komunikaci s matkou a transport potřebných živin. Během předimplantačním období může blastocysta, v důsledku zvýšení počtu buněk a syntéze proteinů, zvětšit svou velikost až tisíckrát a její membrána může zasahovat až do děložních rohů (Blomberg et al. 2008).

2.2.1. Aktivace embryonálního genomu (EGA)

Během prvních několika hodin po oplození musí diferencované zárodečné buňky – oocyt a spermie, splynout a vytvořit totipotentní embryo. Tento proces se nazývá přechod z maternální na embryonální kontrolu vývoje (MZT, maternal-to-zygotic transition) a zajistí, že je nově vznikající embryo schopno následně diferenciovat své buňky v různé buněčné tkáně. Počáteční vývoj a přeprogramování je řízeno především maternálními proteiny a RNA, které byly syntetizovány a uloženy v oocytu v průběhu oogeneze. Genom embrya zůstává transkripčně neaktivní až do období aktivace embryonálního genomu (EGA). Během EGA dochází k rapidnímu nárůstu transkripce a následující vývoj je kontrolován embryonálně. Hlavní vlna EGA (major EGA) nastává v druhově specifický čas. U myši se objevuje v 2buněčném stádiu, u prasete v 4buněčném (Telford et al. 1990), u člověka ve 4 – 8buněčném (Braude et al. 1988) a u skotu v pozdním 8buněčném stádiu (Telford et al. 1990). Neúspěch v regulaci načasování aktivace a volbě exprimovaných genů může vést k zastavení vývoje embrya (Latham & Schultz 2001). Hlavní vlně embryonální transkripce předchází minoritní EGA (minor EGA), kdy je možné ještě v časnějších vývojových stádiích detekovat transkripční aktivitu (Obr. 1). U myši byly nové embryonální transkripty nalezeny dokonce již 7 hodin po formaci prvojader, přičemž první transkripty byly detekovány v samčím prvojádře během G2 fáze prvního buněčného cyklu (Aoki et al. 1997). Nejnovější studie naznačují, že počátek embryonální transkripce není jednotný proces, ale spíše se objevuje ve vlnách v průběhu vývoje.

Obr. 1 Embryonální vývoj a aktivace genomu vybraných modelových organizmů.



Obrázek udává průměrné časové intervaly, po kterých embrya dosáhnou daného vývojového stádia v hodinách po oplození (hpf). Šipkami je označena embryonální aktivace genomu (minoritní a hlavní – majoritní). Údaje převzaty z několika zdrojů (Braude et al. 1988; Telford et al. 1990; Aoki et al. 1997; Holm et al. 1998; Maddox-Hyttel et al. 2007; Jukam et al. 2017; Wamaitha & Niakan 2018).

Aktivace embryonálního genomu je vyvrcholením několika změn, které v embryu během časné embryogeneze nastávají. Mezi ně patří degradace maternálních mRNA a proteinů, nahrazení maternálních transkriptů (housekeeping genů – např. aktin) embryonálními (Minami et al. 2007) a změna struktury chromatinu (Kanka 2003; Vigneault et al. 2009). Degradaci maternálních mRNA a proteinů bude věnována samostatná podkapitola. Reprogramování embryonální genové exprese je umožněno přístupností RNA polymerázy II k promotorům, což je dáno strukturou chromatinu a dostupností transkripčních faktorů (Svoboda 2018).

Krátce po oplození dochází k výměně paternálních protaminů za histony pocházející z cytoplazmy oocytu. Tyto protaminy napomáhaly organizovat DNA spermie do vysoce kompaktních struktur (McLay & Clarke 2003). Od tohoto okamžiku můžeme u některých druhů pozorovat intenzivní demethylaci paternální DNA (např. u myši nebo prasete) (Mayer et al. 2000). I u člověka dochází k demethylaci před EGA, což naznačuje, že se methylace může

podílet na umlčení embryonálního genomu (Guo et al. 2014). U člověka, stejně tak jako u skotu, však dochází pouze k částečné paternální demethylaci (Beaujean et al. 2004). Stejný fenomén však není pozorován u jiných druhů. Například DNA králíka nebo ovce zůstává silně methylována (Beaujean et al. 2004). Z toho vyplývá, že methylace DNA během EGA není konzervovaná mezi jednotlivými druhy (Kelly 2014).

Dále dochází ke změně mezi některými variantami histonů. Například u myši je embryonální varianta histonu H1 přeměněna na somatický během hlavní vlny EGA (Fu et al. 2003). Embryonální varianta H1 formuje pravděpodobně méně stabilní nukleosomy než somatická varianta, díky čemuž se podílí na prostém prostředí časného embrya (Schulz & Harrison 2018). Důležitou modifikací histonů je acetylace, která je obecně spojována s přístupností chromatinu a aktivní transkripcí. Během MZT acetylace histonů stoupá a souvisí s geny aktivovanými během EGA (Li et al. 2014).

Existují dva obecné názory na mechanizmy regulace aktivace genomu. Prvním z nich je teorie „nukleocytoplasmatického poměru“ (N:C ratio) a druhý jsou „maternální hodiny“ (maternal-clock). Nukleocytoplazmatický model vychází ze skutečnosti, že v průběhu buněčného dělení dochází k nárůstu množství jaderného materiálu, zatímco objem cytoplasmy je konstantní. Postupným dělením buněk se tak upravuje N:C poměr a zmírňuje se transkripční represe (Lee et al. 2014; Schulz & Harrison 2018). V tomto modelu jako bariéra pro EGA figurují maternální faktory, jejichž hladina musí být snižována před nástupem transkripce (Lee et al. 2014). Oproti tomu je teorie „maternálních hodin“ nezávislá na počtu buněčných dělení. Je založená na postupném hromadění maternálních mRNA (faktorů), které se kumulují v latentní formě díky inhibičním RNA-vázajícím proteinům. Aktivaci embryonální transkripce ovlivňuje zvýšení množství nebo aktivity maternálních faktorů, které musí dosáhnout určité hladiny, aby mohly spustit transkripci (Lee et al. 2014; Schulz & Harrison 2018). Tyto dva modely se vzájemně nevylučují a je stále více jasné, že načasování EGA je regulováno více koordinovanými procesy (Schulz & Harrison 2018).

2.3. Degradace maternálních mRNA

Jak již bylo zmiňováno výše, raná embryonální stádia jsou řízena převážně maternálními zásobami mRNA a proteinů, jejichž přítomnost není po aktivaci embryonálního genomu již nadále nutná. Stabilita mRNA je ovlivněna třemi základními rysy: sekvencí mRNA, 7-methylguanylátovou čepičkou na 5‘ konci a délkou 3‘ poly(A) konce (Yartseva & Giraldez 2015). Modifikace a sekvence mRNA kódují rozpoznávací místa pro faktory, které pozitivně

nebo negativně regulují stabilitu mRNA, translaci a lokalizaci pro umožnění specifické genové exprese (Fu et al. 2014). Vazebné faktory vedou buď k endonukleolytickému štěpení z obou nechráněných konců mRNA, nebo ke stimulaci deadenylace vedoucí k odstranění čepičky některých mRNA (Decker & Parker 1994) a slouží tak jako prostředek regulující rychlost degradace mRNA (Wahle & Winkler 2013). Poly(A) konec orientovaný na 3‘ konci mRNA je vázaný poly(A)-vazebnými proteiny ke stabilizaci 3‘ konce mRNA (Bernstein et al. 1989) a zároveň interaguje s translačním iniciačním faktorem eIF4G navázaným na 5‘ čepičce ke stimulaci translace (Weill et al. 2012). mRNA s čepičkou jsou chráněné před rozpadem a její hydrolýza může vézt k destabilizaci celé mRNA (Cowling 2009).

Experimentálně bylo prokázáno, že degradace maternálních mRNA a proteinů v koordinaci s EGA je nezbytným krokem pro časný embryonální vývoj myši (Tripurani et al. 2013). Předpokládá se, že za degradaci maternálních mRNA zodpovídají mikroRNA (miRNA). MiRNA jsou rodina malých, jednovláknových nekódujících RNA, které jsou dlouhé 21 – 23 nukleotidů a jsou evolučně konzervovány (Ambros 2004). Fungují jako negativní regulátory genů na posttranslační úrovni. Váží se na 3’UTR region cílové mRNA pomocí párování s jejími bázemi a následně mRNA rozštěpí nebo inhibují translaci (Bartel 2004). U myši dochází k destabilizaci většiny mRNA před EGA. Zrání oocytu mezi fázemi GV a MII spouští destabilizaci téměř 3000 různých mRNA. Tyto transkripty jsou zahrnuty především v produkci ATP, například oxidativní fosforylaci a biosyntéze ubiquinonu, které reflektují změny v metabolických potřebách oocytu během zrání. Degradace maternálních mRNA během maturace oocytu je pečlivě regulovaná. Důkazem toho je, že přes 9200 mRNA zůstává v tomto období stabilní (Su et al. 2007). Téměř 2300 maternálních mRNA je odstraněno ihned po oplození a dalších téměř 500 mRNA je degradováno v 2buněčném stádiu (Hamatani et al. 2004). Yokoi et al. (1993) popisují, že v 2buněčném myším embryu lze detekovat pouze 10 – 20 % mRNA z původního množství mRNA v neoplozeném oocytu. Walser & Lipshitz (2011) uvádí, že přibližně 30 % maternálních mRNA je degradováno v průběhu oogeneze myši a dalších 30 % je sice v tomto období deadenylováno, ale odstraněno až v čase MZT. Také v časném embryonálním vývoji lidských embryí hraje eliminace maternálních mRNA nezbytnou roli. Bylo prokázáno, že během druhého dne po oplození je odstraněno asi 1700 mRNA a mezi druhým a třetím dnem dalších přibližně 700 mRNA (Zhang et al. 2009). Tyto výsledky ukazují, že i v lidském vývoji lze objevit dramatické změny ve skladbě mRNA objevující se ve vlnách, ve kterých se vždy degradují pouze určité skupiny mRNA (v 2buněčném stádiu především mRNA kontrolující buněčný cyklus nebo regulující transkripci,

v pozdějších stádiích mRNA regulující fosforylaci proteinů nebo buněčnou morfogenezi) (Yan et al. 2013). Zdá se, že degradace maternálních mRNA je nezbytná pro následující vývin. U některých lidských embryí, která zastavila svůj vývoj, bylo sice možné detekovat EGA, ale selhalo u nich snižování exprese maternálních transkriptů (Yartseva & Giraldez 2015). Walser & Lipshitz (2011) popisují několik funkcí odstranění maternálních transkriptů. První je permisivní a instruktážní funkce, kdy eliminace maternálních mRNA může zprostředkovat transkripci jejich zygotických protějšků, umožňující časově a prostorově omezenou kontrolu vývoje (De Renzis et al. 2007). Další způsob může být odstranění maternálních transkriptů, které byly kriticky nutné v období oogeneze, ale pro následující embryonální vývoj již nejsou potřebné (Pan et al. 2005).

2.4. Degradace maternálních proteinů

Jak bylo uvedeno, degradace maternálních mRNA ještě není úplně objasněným úsekem embryonálního vývoje, degradace maternálních proteinů je však prozkoumána ještě daleko méně. Jako nejpravděpodobnější způsob, kterým dochází k degradaci maternálních proteinů se jeví degradace pomocí ubiquitin-proteasomového systému (UPS) a autofagie. Nejnovější výzkumy naznačují, že degradace maternálních proteinů během embryonálního vývoje není hromadný proces. Pravděpodobně je degradace jednotlivých proteinů spíše kontrolována odděleně.

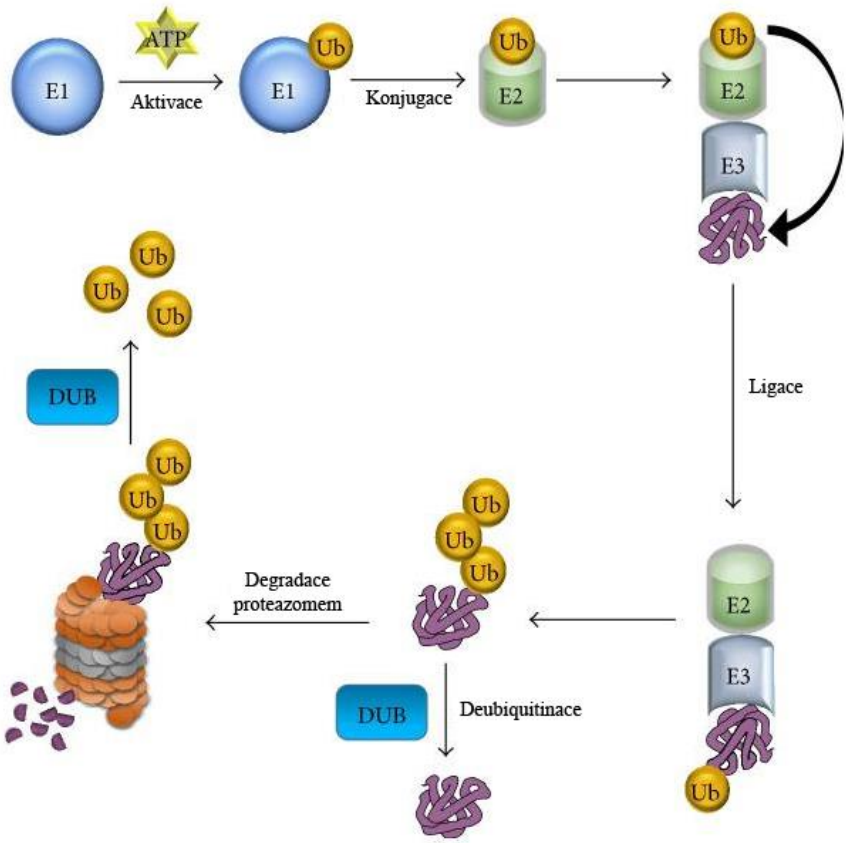
2.4.1. Ubiquitin – proteazomový systém (UPS)

Pomocí UPS dochází k regulované degradaci až 90 % krátkodobých proteinů (Ciechanover et al. 1984). Tento způsob degradace je vysoce specifický a je primárně určen k degradaci endogenních proteinů. Proteolýza zprostředkovaná UPS hraje důležitou roli ve spoustě základních buněčných procesů. Ovlivňuje buněčný cyklus a dělení, diferenciaci a vývoj, je zahrnuta v buněčné odpovědi na stres, morfogenezi neuronové sítě, modulaci povrchových receptorů buňky, iontových kanálů, opravy DNA, regulaci transkripce, dlouhodobé paměti, cirkadiálních rytmech, regulaci imunity a biogenezi organel (Fang & Weissman 2004). Defekty v ubiquitinaci jsou naopak spojovány s mnoha onemocněními, mezi které patří například vývojové abnormality, neurodegenerativní onemocnění (Alzheimerova choroba, Parkinsonova choroba,…), Downův syndrom, rakovina a problémy s autoimunitou (Weissman 2001). Na protein, určený k degradaci, je kovalentně navázán ubiquitin, vysoce evolučně konzervovaný, 76 aminokyselin dlouhý polypeptid (Fang & Weissman 2004). Takto označený protein je následně rozeznán 26S proteazomem a degradován (Glickman & Ciechanover 2002). Pro správnou detekci cílového proteinu proteazomem je u eukaryot nutné,

aby došlo k polyubiquitinaci, tedy navázání několika ubiquitinů v řadě. Minimální počet navázaných ubiquitinů jsou čtyři. V opačném případě, tedy při vazbě méně než čtyř ubiquitinů, může být vyvolán funkčně odlišný signál a dojde například ke spuštění endocytózy nebo regulaci histonů (Hicke 2001). Záleží také, kde přesně na cílovém proteinu je ubiquitin navázán. Pro degradaci proteinu je nezbytné, aby byl ubiqutin vázán na lysinu daného proteinu. Weissman (2001) uvádí, že pro odstranění proteinu pomocí proteasomu dochází nejčastěji k vazbě na Lys-48 nebo Lys-29. Zatímco vazba na jiném lysinovém zbytku, např. Lys-63 může fungovat jako signál pro opravu DNA, endocytózu nebo autofagii (Thrower et al. 2000; Tan et al. 2008). Množství proteazomů je regulováno především na transkripční úrovni jeho podjednotek (Livneh et al. 2016). Pokles v proteozomální aktivitě vede ke snížení exprese mRNA proteozomálních genů a následně i formaci proteazomu *de novo* (Meiners et al. 2003). Proteazomy se v savčích buňkách primárně nachází v cytosolu, ale jsou spojovány i s cytoskeletárními elementy, endoplasmatických retikulem, jádrem a plasmatickou membránou. Nicméně poměr proteazomů spojovaných s jednotlivými organelami závisí na druhu buňky (Wójcik & DeMartino 2003).

Degradace v proteazomu je několika stupňový proces a vyžaduje spoluúčast tří enzymatických komplexů: E1 – ubiquitin aktivujícího, E2 – ubiquitin konjugujícího enzymu a E3 – ubiquitin ligázy (Glickman & Ciechanover 2002). Zatímco můžeme rozeznat pouze jeden typ E1 enzymu a něco přes 40 typů E2 enzymů, existuje více než 500 druhů E3 ligáz, které umožňují výběr z širokého spektra cílových proteinů (Fuchs 2005). Proces navázání ubiquitinu na cílový enzym se nazývá ubiquitinace, detailně je znázorněn na obrázku 2 (Obr. 2) a probíhá v následujících krocích: nejprve musí dojít k aktivaci ubiquitinu pomocí E1 enzymu za přítomnosti ATP a následné tvorbě E1-ubiquitin komplexu. Tento komplex je rozeznán E2 enzymy, na které je ubiquitin přenesen. Každý E2 enzym je schopný se vázat s větším množstvím E3 enzymů a E3 enzymy jsou zodpovědné za finální volbu cílového proteinu a specifičnost. E2 enzym s ubiquitinem je navázán na E3 enzym, který zprostředkuje konečnou vazbu ubiquitinu na protein určený k degradaci (Pickart 2001; Weissman 2001). Takto označený protein je následně rozeznán 26S proteazomem, velkým proteázovým komplexem. 26S proteazom se skládá z proteolytického jádra a regulační čepičky (Glickman et al. 1998). Substrát je zde rozkládán za přítomnosti ATP a nerozložené polypeptidové řetězce jsou přesunuty do katalytického těla proteazomu, kde jsou pomocí proteáz degradovány na krátké peptidy (Baumeister et al. 1998).

Obr. 2 Schéma ubiquitinace.



Ubiquitinace je katalyzována pomocí enzymů E1, E2 a E3, které zprostředkují navázání molekuly ubiquitinu (Ub) na lyzinový zbytek proteinového substrátu. Polyubiquitinový řetězec navázaný na Lys-48 cílového proteinu zabezpečuje degradaci pomocí 26S proteazomu. DUB (deubiquitinační) enzymy ubiquitin odstraňují a recyklují (převzato a upraveno z Suresh et al. (2016).

Ubiquitin musí být z cílového proteinu odstraněn ještě před tím, než vstoupí do proteolytického jádra proteazomu. Jeho odstranění zabezpečují deubiquitinační enzymy (DUBs) dělící se na ubiquitin procesující (UBP) a ubiquitin karboxy-terminální hydrolázy (UBH). Obecně lze říci, že UBP odstraňují ubiquitin z polyubikvitinovaných proteinů, zatímco UBH odstraňují malé adukty z ubiquitinu a obnovují jeho volné monomery. Tyto deubiquitinační enzymy jsou thiol proteázy, které štěpí vazbu mezi ubiquitinem a substrátem (Weissman 2001). Tímto způsobem DUBs kontrolují nejen degradaci substrátu, ale také recyklaci ubiquitinu k pozdějšímu využití. Bylo prokázáno, že umlčení nebo inhibice DUB enzymů ovlivnila preimplantační vývoj (Susor et al. 2010). Opoždění vývoje bylo pravděpodobně zapříčiněno především nedostatkem volných monoubiquitinů (Mtango et al. 2014). Například umlčení USP36, který je důležitý pro vznik ribozomů a zpracování RNA, způsobuje snížení translace mRNA u myších morul (Fraile et al. 2018). DUB enzymy hrají

důležitou roli i při oplození v ochraně proti polyspermii. Především pak ubiquitin C-terminální hydrolázy (UCHLs), které jsou jedny z nejdůležitějších DUB enzymů (Susor et al. 2010). Jejich nejvýznamnějšími zástupci jsou UCHL1 a UCHL3, které jsou nezbytné pro normální zrání oocytů a oplození (Mtango et al. 2014). Obzvláště UCHL1 je vysoce exprimován v oocytech prasete a skotu. Je lokalizován v kortexu oocytu, pravděpodobně z toho důvodu, aby se mohl podílet na zrání kortikálních granulí nutných k pozdější ochraně proti polyspermii (Susor et al. 2010). UCHL3 je lokalizován na dělícím vřeténku a je důležitý pro správné oddělení pólového tělíska (Mtango et al. 2014). Nesprávná exprese UCHLs během oogeneze a oplození způsobuje abnormální embryonální vývoj, přičemž většina defektů je viditelná během kompaktace moruly a formování blastocysty (Mtango et al. 2012).

Degradace proteinů pomocí UPS reguluje velké množství buněčných procesů, zahrnující například remodelaci chromatinu a methylaci H2B (Osley 2004), průběh buněčného cyklu a transkripci (Lipford et al. 2005). UPS reflektuje také kvalitu embryí (Mtango & Latham 2007). Z těchto důvodů se předpokládá, že se UPS podílí i na degradaci maternálních proteinů (Verlhac et al. 2010). Není však zatím jasné, které E3 ligázy se na této degradaci podílejí, ani to, kdy jsou které substráty odstraňovány.

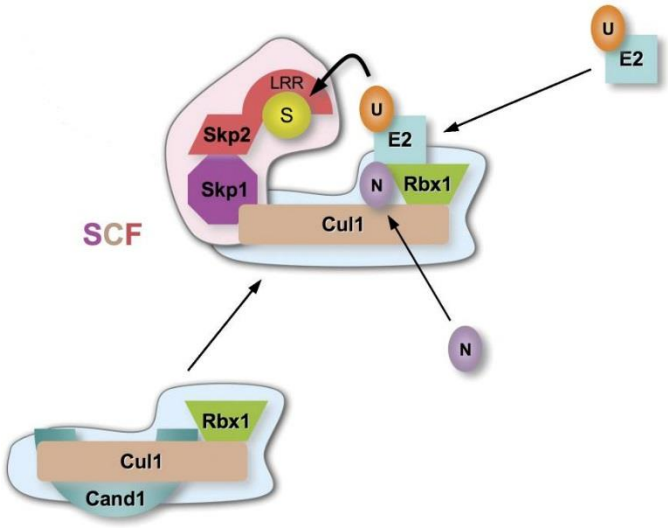
Jak již bylo uvedeno, specificitu ubiquitin – dependentní proteolýzy udávají stovky E3 ligáz, které jsou schopny rozeznat vazebné domény substrátů (Varshavsky 1991). E3 enzymy se na základě charakteristických motivů rozdělují do dvou základních rodin: RING a HECT. HECT ligázy sami o sobě fungují jako přenašeči ubiquitinu. Jejich katalytická doména formuje vazbu s ubiquitinem, aby ho přenesla k vazbě na substrát (Scheffner et al. 1993). RING ligázy naopak nemají žádnou katalytickou aktivitu, ale využívají Zn-vazebný RING strukturální motiv, aby nasměrovali E2 enzym ke specifickému substrátu (Borden 2000). Pravděpodobně nejrozmanitější rodinou jsou RING E3 ligázy, mezi které se u lidí řadí okolo 400 proteinů, zatímco HECT domén je přibližně 40 (Hindley et al. 2011). Mezi nejznámější RING ligázy patří APC/C (Anaphase Promoting Complex/Cyclosome) a SCF ligázy. SCF (Skp1-Cullin-F box) komplex se skládá z proteinu SKP1, CUL1, RBX1 a jednoho zástupce z rodiny F-BOX proteinů. Aktivace SCF komplexu je řízena neddylací. Jedná se o posttranskripční modifikaci, kterou zprostředkovává ubiquitinu-podobný protein NEDD8. Neddylace je proces velice podobný ubiquitinaci. Podílejí se na něm také tři enzymatické komplexy: enzym E1, E2 a E3, které zabezpečují aktivaci NEDD8 a jeho přenos a kovalentní vazbu na cílový substrát. Jako substráty neddylace byly identifikovány proteiny patřící do rodiny cullinů (Huang et al. 2007), přes jejichž modifikaci může NEDD8 ovlivňovat funkci celého SCF komplexu (Pan et al.

2004). Inhibice neddylace, potažmo celého SCF komplexu, je obstarána pomocí proteinu CAND1 (Cullin-Associated and Neddylation-Dissociated 1 Protein). CAND1 se váže na CUL, díky čemuž dochází k odstranění proteinu NEDD8 pomocí COP9/signalozomu, velkého multiproteinového komplexu, který má specifickou isopeptidázovou aktivitu (Bornstein et al. 2006). Vazbou CAND1 dochází k regulaci formace SCF komplexu zabráněním navázání F-box proteinů a SKP1 na CUL1. Naopak, neddylace CUL1 způsobuje disociaci CAND1 (Zheng et al. 2002a).

2.4.2. SCF komplex

SCF E3 ligázy jsou nejlépe charakterizovanou skupinou RING E3 ligáz obsahujících Cullin. Jak již bylo uvedeno, skládají se z Cul1, Rbx1, Skp1 a jednoho zástupce z rodiny F-box proteinů (Obr. 3). Cullin1 a Rbx1 vytváří katalytické jádro, které váže příslušný E2 enzym s navázaným ubiquitinem. Skp1 slouží jako adaptor umožňující připojení F-box proteinu na Cullin1 a F-box protein určuje substrátovou specifitu (Zheng et al. 2002b).

Obr. 3 Schéma SCF komplexu



SCF komplex složený z Cul1, Rbx1, Skp1 a jednoho zástupce z rodiny F-box proteinů (v tomto případě Skp2 protein). Na obrázku je znázorněn neaktivní komplex Cul1-Rbx1 inhibovaný vazbou na Cand1. Po navázání proteinu Nedd8 (N) dochází k formování komplexu vazbou jeho ostatních členů, E2 enzym přináší ubiquitin (U) a na LRR úsek (úsek bohatý na leucin) F-box proteinu nasedá substrát, který je následně ubiquitinován (převzato a upraveno z Zhang et al. (2008b).

Oporou a nosnou jednotkou celého komplexu je **Cullin1 (Cul1)**, který zároveň řídí aktivitu celého komplexu vazbou na CAND1 (kdy je SCF komplex neaktivní) nebo NEDD8 (kdy se CAND1 disociuje, CUL1 se váže na SKP1 a formuje tak aktivní SCF komplex) (Liu et

al. 2002). Cul1 je u skotu exprimován ve dvou odlišných formách ze dvou rozdílných genů, obou lokalizovaných na chromozomu 4, avšak v odlišných regionech. Jedna forma byla identifikována jako embryonální Cul1, který je exprimovaný od pozdního 8buněčného stádia po stádium blastocysty. Tato varianta je exprimována v somatických buňkách. Druhá varianta je nazývána Cul1-like neboli maternální Cullin1. Je exprimovaný u oocytů ve stádiu MII až po embrya v časném 8buněčném stádiu a s embryonální variantou Cul1 sdílí 83 % homologii (Kepkova et al. 2011). O funkci Cul1 v průběhu časného embryonálního vývoje savců byly publikovány dvě studie. Dealy et al. (1999) a Wang et al. (1999) prokázali nezbytnost Cul1 v průběhu embryogeneze myši, kdy po jeho inhibici došlo k zastavení vývoje. Pravděpodobně se podílí na hatchingu a následujících procesech spojených s uhnízděním embrya v děloze, jelikož exprese mRNA Cul1 prokazatelně roste od stádia blastocysty (Hwang et al. 2004). Dále hraje roli v diferenciaci buněk trofodermu a vývoji placenty (Zhang et al. 2013). Jeho detailní role v časném vývoji však nebyla prozatím specifikována.

Skp1 (S-phase Kinase-Associated Protein 1) je stabilní protein, který váže katalytické jádro SCF komplexu k F-box proteinu (Bai et al. 1996; Skowyra et al. 1997; Zheng et al. 2002a). Jeho funkce jsou spjaty především s SCF komplexem, jeho exprese je však spojená i s rozvojem Parkinsonovy choroby (Mandel et al. 2012; Rhodes et al. 2013) nebo lymfomy (Piva et al. 2002).

Rbx1 (RING Box Protein-1; ROC1, Regulator of Cullins) společně s Cul1 tvoří katalytické jádro komplexu. Mimo Cul1 je Rbx1 schopný interagovat i s ostatními culliny (Ohta et al. 1999). Obsahuje RING finger doménu, která váže E2 enzym a zprostředkovává neddylace Cul (Morimoto et al. 2003). Je zodpovědný za normální progres meiózy u myši a *Xenopa* (Gutierrez et al. 2006; Marangos et al. 2007; Zhou et al. 2013). Jeho umlčení vede k zastavení embryonálního vývoje myši, *Caenorhabditis elegans a Drosophila melanogaster* (Sasagawa et al. 2003; Moore & Boyd 2004; Tan et al. 2009; Jia et al. 2011) a způsobuje mezery v dvouvláknové struktuře DNA, zastavení buněčného cyklu v G2 fázi, aneuploidii a jeho zvýšená exprese byla detekovaná při rakovinném bujení (Jia et al. 2009).

Univerzálnost SCF ligáz je zajištěna pomocí **F-box** proteinů, které jsou schopné rozpoznávat velké množství substrátů. Rozsáhlost této skupiny dokazuje i skutečnost, že jen u lidí bylo popsáno minimálně 38 těchto proteinů (Kipreos & Pagano 2000). F-box proteiny jsou krátkodobě žijící proteiny, které jsou destabilizovány jejich inkorporací do SCF komplexu (Wirbelauer et al. 2000). F-box proteiny obsahují F-box motiv umožňující interakci s jinými

proteiny, díky kterému připojují specifické substráty k ubiquitinačnímu komplexu (Bai et al. 1996). Mnoho F-box proteinů je schopno interakce s daným substrátem pouze v případě, že byl substrát posttranslačně modifikován, což vytváří další způsob regulace degradace proteinů. Přestože mezi tyto modifikace nejčastěji patří fosforylace, bylo prokázáno, že některé substráty F-box proteinů musí být také acetylované, glykosylované nebo nitrované (Hwang et al. 2010). Například degradace cyklinu E pomocí SCF^{Fbw7} komplexu je provedena pouze po předchozí fosforylaci (Ye et al. 2004).

2.4.3. Další způsoby degradace proteinů – autofagie, endocytóza

Autofagie je, po UPS, druhým nejlépe prozkoumaným procesem k degradaci proteinů. Autofagií jsou degradované i dalších molekuly, například lipidy, malé organely a další molekuly přítomné v cytoplazmě. Zároveň se podílí i na degradaci maternálních mRNA v průběhu časného vývoje (Chi et al. 2017). Dále je autofagie potřebná i pro recyklaci aminokyselin potřebných pro *de novo* syntézu proteinů (Tsukamoto et al. 2008). Celý proces degradace začíná vytvořením autofagozomů, do kterých je pohlcena část cytoplazmy. Autofagozom následně splyne s lysozomy, ve kterých jsou proteiny degradovány na jednotlivé aminokyseliny (Boya et al. 2005). Autofagie je regulovaná hlavně pomocí mTOR a Atg proteinů (He & Klionsky 2009). Podílí se i na regulaci proteinů spjatých s pluripotencí – Oct4, Nanog a Sox2 (Lee et al. 2011). Vyšší aktivita autofagie pravděpodobně souvisí s vyšší kvalitou embryí. Bylo prokázáno, že myší embrya s vyšší aktivitou autofagie měla v 4buněčném stádiu vyšší kvalitu (Tsukamoto et al. 2014), prasečí oocyty a bovinní preimplantační embrya s indikovanou autofagií vykazovala zlepšení vývojové kompetence (Song et al. 2012; Lee et al. 2016). Autofagie je propojená s ubiquitinací, jelikož může degradovat proteinové komplexy, které nebyly dokonale rozloženy pomocí UPS nebo organelami (Tsukamoto & Tatsumi 2018). Jak bylo uvedeno výše, autofagie může být spuštěna i v případě, že se ubiquitin naváže na daný lysin. Konkrétně vazba na lysin 63 spustí místo ubiquitinace autofagii, endocytózu nebo opravu DNA (Thrower et al. 2000; Tan et al. 2008). Během degradace poničených mitochondrií spolupracuje autofagie s E3 ubiquitin ligázami PARKIN a MUL1, které ubiquitinují protein membrány mitochondrií. V tomto procestu se stává autofagie selektivní, protože UPS označí mitochondrie pro lysozomální degradaci (Rojansky et al. 2016). Dalším případem spolupráce těchto dvou systémů je během paternální mitofágie po oplození (Hajjar et al. 2014; Rojansky et al. 2016). Autofagie a UPS tak spolu mohou spolupracovat, aby zajišťovali správnou degradaci nebo naopak uchování maternálních proteinů v průběhu preimplantačního vývoje.

Endocytóza je potřebná pro velkou škálu buněčných funkcí, včetně přenosu neuronálních, metabolických a proliferačních signálů, příjem mnoha základních živin a regulace buněčné homeostázi (Mellman 1996). Předtím než jsou proteiny degradovány pomocí lysozomů, procházejí přes dvě morfologicky a biochemicky odlišné organely – časný a pozdní endozom (Geli & Riezman 1998). Lysozomální degradace nemusí být jediným osudem internalizovaných proteinů. Mnoho receptorů je z časného endozomu recyklováno zpět do plazmatické membrány. V některých specializovaných typech buněk je tato recyklace využita také pro prezentaci antigenu nebo recyklaci složek synaptických vezikul (Galan et al. 2001).

2.5. Degradace vybraných proteinů v průběhu oogeneze a preimplantačního vývoje

SCF komplex patří mezi nejdůležitější SCF ligázy. Regulací významného množství různých substrátů ovlivňuje velkou řadu buněčných procesů. Mezi ně patří například progrese buněčného cyklu, přenos signálů, transkripce, DNA replikace, modulace virů, vývoj, cirkadiální rytmy a kontrola kvality proteinů (Zhou et al. 2013). Mezi substráty SCF komplexu patří například cyklin A, D, E, Cdc25A, p21, p27, p53, c-Jun, IκB, β-catenin, Myc a mnoho dalších (Jia et al. 2009). Díky degradaci cyklinu E nebo proteinu p27 se SCF komplex podílí na regulaci buněčného cyklu. SCF komplex obsahující FBW7 (tedy SCF^{FBW7} komplex), který patří mezi nejprostudovanější F-box proteiny, degraduje již zmiňované onkogenní proteiny: cyklin E, c-Myc, c-Jun a MCL1, což z SCF^{FBW7} činí jednoho z nejlépe zavedených supresorů nádorového bujení (Shimizu et al. 2018).

SCF komplex se podílí na degradaci některých důležitých faktorů již v období oogeneze. Po inhibici RBX1 proteinu dochází k akumulaci Emi1, inhibitoru APC/C komplexu, nezbytného pro maturaci myších oocytů. Emi1 je degradován pomocí $SCF^{\beta TrC}$ komplexu a při jeho akumulaci dochází k zastavení vývoje v metafázi prvního meiotického dělení (metafáze I) a snížení úspěšnosti vydělení prvního pólového tělíska (Zhou et al. 2013). Jin et al. (2019) před nedávnem popsali protein FBXO30, nový člen F-box rodiny, který se hojně vyskytuje v počátcích oogeneze myších oocytů. Jeho exprese postupně klesá po metafázi I. Bylo zjištěno, že je nezbytný pro oddělování chromozomů, které reguluje pomocí SLBP proteinu (Stem-Loop-Binding Protein). Při inhibici FBXO30 dochází k hromadění SLBP a následnému selhání v segregaci chromozomů (Jin et al. 2019).

Přestože u myši dochází k velkému poklesu v množství proteinů mezi stádii MII a zygotami (Wang et al. 2010), ne všechny proteiny jsou před EGA odstraněny. Některé proteiny

jsou uchovávány během celého preimplantačního vývoje (Ohsugi et al. 2008; Toralová et al. 2012). Aby maternální proteiny přetrvaly během preimplantačního vývoje a mohly např. regulovat embryogenezi ještě po EGA, vytvářejí komplexy (Li et al. 2013). Mezi takové proteiny patří například proteiny *ZP* (*Zona Pellucida* Sperm-Binding Proteins), SCMC (Subcortical Maternal Complex) a další (Lu et al. 2017). Jejich exprese je pouze maternální a příslušná mRNA je degradována v době EGA, tyto proteiny jsou nicméně skladovány až do stádia blastocysty (Bebbere et al. 2016). Další variantou modifikace proteinů je dočasné maskování, např. proteinu CENPE během zrání oocytů. Je pravděpodobné, že existuje více proteinů, které formují komplexy, jsou maskovány nebo případně jinak modifikovány, aby byly uchovány během celého preimplantačního vývoje (Duesbery et al. 1997). Jiné proteiny jsou exprimovány během časné embryogeneze beze změny (Allard et al. 2002; Toralová et al. 2009). Některé maternální proteiny však musí být z embrya odstraněny, aby následující vývoj mohly kontrolovat jejich embryonální verze. Mezi proteiny, které musí být u myši odstraněny před hlavní vlnou EGA, patří TAB1 (TGF-beta Activated Kinase 1 Binding Protein). Tento protein je z embrya odstraněn pomocí RNF114 (Ring finger protein 114) E3 ubiquitin ligázy a v případě jeho nahromadění dochází k zastavení embryonálního vývoje v 2buněčném stádiu (Yang et al. 2017). Další proteiny, jejichž degradace před hlavní vlnou EGA je pro další vývoj nezbytná, byly nalezeny především u rodu *Xenopus*. Jedná se například o CDC25A (Shimuta et al. 2002), CDC6 (Cell Division Control Protein), Treslin, RecQL4 (Sun et al. 2014) a Drf1/DBF4B (Dumbbell-Forming 4) (Collart et al. 2013). U *Caenorhabditis elegans* pak dochází k odstranění proteinů MEI-I a MEI-2. Oba tyto proteiny jsou důležité pro meiotické dělící vřeténko. Ihned po dokončení meiózy tak musí být z oocytu odstraněny, aby mohlo následně vzniknout velké mitotické dělící vřeténko, které se svou morfologií od toho meiotického liší. MEI-I a MEI-II jsou odstraňovány pomocí E3-ligázy obsahující Cul3 (DeRenzo & Seydoux 2004; Verlhac et al. 2010). Po inhibici UPS nebo jeho součástí u savců dochází k opoždění minoritní i hlavní vlny EGA (Shin et al. 2010; Shin et al. 2013; Shimizu et al. 2014; Yang et al. 2017). To naznačuje, že degradace proteinů je vysoce specifická a odstranění některých z maternálních proteinů je pro další vývoj nezbytné. Zbývá jen určit, o které proteiny se jedná a jakým způsobem jsou z embrya odstraněny.

3. Hypotéza a cíle

Hypotéza

Degradace přinejmenším některých maternálních proteinů pomocí ubiquitin – proteazomového systému je nezbytným předpokladem k aktivaci embryonálního genomu (EGA) savců.

Cíl práce

Cílem práce byla identifikace dějů, které jsou nezbytné pro správný průběh preimplantačního vývoje skotu a zahájení embryonální genomové aktivace. Výzkum se zaměřoval zejména na úlohu proteazomového systému, především SCF komplexu, a jeho vliv na degradaci vybraných maternálních proteinů skotu.

4. Publikované práce

4.1. Charakterizace SCF komplexu v průběhu preimplantačního vývoje skotu

Prozatím není jasné, jakým způsobem jsou z embryí odstraněny zbytky maternálních proteinů. Předpokládali jsme, že na jejich degradaci by se mohl podílet SCF komplex, který patří mezi nejčastější ubiquitin-ligázy zprostředkovávající vazbu ubiquitinu na protein určený k degradaci. Hlavním cílem této práce bylo tedy sledování exprese jednotlivých členů SCF komplexu a jejich lokalizace v rámci jednotlivých vývojových stádií skotu. Pomocí kvantitativní RT-PCR metody byla sledována exprese mRNA Cul1, Skp1 a Rbx1. Exprese všech tří neměnných členů SCF komplexu začala v častém 8buněčném stádiu postupně stoupat. Pro odhalení, kdy přesně začíná exprese Cul1, Skp1 a Rbx1 z embryonálního genomu byla využita kultivace s α-amanitinem, který inhibuje RNA polymerázu II a způsobí tak zastavení transkripce. Exprese Rbx1 mRNA začíná až v pozdním 8buněčném stádiu, avšak exprese Cul1 a Skp1 se objevuje již v 4buněčném a časném 8buněčném stádiu, což naznačuje jejich důležitost pro EGA a následující vývoj. Následně jsme sledovali expresi proteinů jednotlivých členů SCF komplexu a jejich lokalizaci v rámci jednotlivých stádií. Množství proteinu CUL1 postupně rostlo od MII oocytů po moruly, byl přítomen v jádře a cytoplazmě během celého vývoje. Hladina SKP1 rostla od MII po 4buněčné stádium, následně však opět začala signifikantně klesat. Protein byl lokalizován v průběhu celého vývoje v cytoplazmě a částečně i v *zona pellucida*. Analýza RBX1 proteinu, i přes pozorovatelný nárůst od MII do 4buněčného stádia a následný opětovný pokles, neodhalila žádný signifikantní rozdíl v jeho množství v průběhu časného vývoje a protein byl opět lokalizován u všech vývojových stádií.

Následující částí našeho výzkumu bylo sledování aktivity SCF komplexu během preimplantačního vývoje. Pomocí metody PLA (*in situ* Proximity Ligation Assay), která fluorescenčně označí proteiny, které se nachází ve vzdálenosti do 40nm (Bagchi et al. 2015), jsme sledovali vazbu proteinu Skp1 na Cul1. Díky jejich vzájemné interakci dojde i k následné aktivaci celého komplexu. Mezi jednotlivými embryonálními stádii nebyl zaznamenán žádný statisticky významný nárůst v aktivitě tohoto komplexu. Nejintenzivnější signál byl pozorován u MII oocytů, ani ten však nebyl oproti preimplantačním embryím signifikantní. Ve stádiu blastocysty se však projevilo nerovnoměrná lokalizace aktivního SCF komplexu. V oblasti vnitřní buněčné masy byla aktivita tohoto komplexu snížena na rozdíl od oblasti

trofoektodermu, což naznačuje důležitou roli SCF komplexu pro formování extraembryonální tkáně.

Publikace:

- Benesova V, **Kinterova V**, Kanka J, Toralova T. 2016. Characterization of SCF-Complex during Bovine Preimplantation Development. PLOS ONE **11**(1):e0147096.
- Benesova V, **Kinterova V**, Kanka J, Toralova T. 2017. Potential Involvement of SCF-Complex in Zygotic Genome Activation During Early Bovine Embryo Development. In: Lee K. (eds) Zygotic Genome Activation. Methods in Molecular Biology **1605**. Humana Press, New York, NY.

PLOS | ONE

RESEARCH ARTICLE

Characterization of SCF-Complex during Bovine Preimplantation Development

Veronika Benesova[1,2]*, Veronika Kinterova[1,3], Jiri Kanka[1], Tereza Toralova[1]

1 Laboratory of Developmental Biology, Institute of Animal Physiology and Genetics Academy of Science of Czech Republic, v.v.i., Libechov, Czech Republic, **2** Faculty of Science, Charles University in Prague, Prague, Czech Republic, **3** Department of Veterinary Sciences, Czech University of Life Sciences in Prague, Prague, Czech Republic

* benesova@iapg.cas.cz

OPEN ACCESS

Citation: Benesova V, Kinterova V, Kanka J, Toralova T (2016) Characterization of SCF-Complex during Bovine Preimplantation Development. PLoS ONE 11(1): e0147096. doi:10.1371/journal.pone.0147096

Editor: Srinivasa M Srinivasula, IISER-TVM, INDIA

Received: August 19, 2015

Accepted: December 30, 2015

Published: January 29, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Czech Science Foundation (GACR 13-24730P) (TT) and Grant Agency of Charles University in Prague (GAUK 1280214) (VB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

The degradation of maternal proteins is one of the most important events during early development, and it is presumed to be essential for embryonic genome activation (EGA), but the precise mechanism is still not known. It is thought that a large proportion of the degradation of maternal proteins is mediated by the ubiquitin-proteolytic system. In this study we focused on the expression of the Skp1-Cullin1-F-box (SCF) complex, a modular RING-type E3 ubiquitin-ligase, during bovine preimplantation development. The complex consists of three invariable components—Cul1, Skp1, Rbx1 and F-box protein, which determines the substrate specificity. The protein level and mRNA expression of all three invariable members were determined. *Cul1* and *Skp1* mRNA synthesis was activated at early embryonic stages, at the 4c and early 8c stage, respectively, which suggests that these transcripts are necessary for preparing the embryo for EGA. CUL1 protein level increased from MII to the morula stage, with a significant difference between MII and L8c, and between MII and the morula. The CUL1 protein was localized primarily to nuclei and to a lesser extent to the cytoplasm, with a lower signal in the inner cell mass (ICM) compared to the trophectoderm (TE) at the blastocyst stage. The level of SKP1 protein significantly increased from MII oocytes to 4c embryos, but then significantly decreased again. The localization of the SKP1 protein was analysed throughout the cell and similarly to CUL1 at the blastocyst stage, the staining was less intensive in the ICM. There were no statistical differences in RBX1 protein level and localization. The active SCF-complex, which is determined by the interaction of Cul1 and Skp1, was found throughout the whole embryo during preimplantation development, but there was a difference at the blastocyst stage, which exhibits a much stronger signal in the TE than in the ICM. These results suggest that all these genes could play an important role during preimplantation development. This paper reveals comprehensive expression profile, the basic but important knowledge necessary for further studying.

Introduction

At the very beginning of early embryogenesis, all the mRNAs and proteins are of maternal origin and development is driven by these maternal reserves. As development proceeds, these reserves are stepwise degraded and the embryo takes over control of development. The degradation of maternal mRNAs is a gradual process, which peaks around the major wave of embryonic genome activation (EGA), and results in the degradation of the vast majority of maternal mRNAs at this time [1]. However, little information about protein degradation is available. Presumably, the degradation of maternal protein is essential for the start of embryonic genome transcription, but the precise mechanism of EGA initiation is still not known. The process is certainly not as rapid as mRNA degradation, and some of the proteins remain even after EGA [2–4]. It is thought that a large proportion of the degradation of maternal proteins is mediated by the ubiquitin-proteolytic system [5–8]. The ubiquitin-proteasome pathway directs protein degradation, and thus serves as a regulatory mechanism of various cellular processes. The ubiquitination of proteins is a stepwise process, which is managed by the cooperation of three enzyme complexes: E1 –ubiquitin activating enzyme; E2 –ubiquitin-conjugating enzyme and E3 –ubiquitin ligase [9]. The E3 ligases transfer ubiquitin from E2 to the substrate protein, and their plurality (caused by F-box protein variability) enables the specific labelling of various proteins.

In this study we have focused on the expression of the Skp1-Cullin1-F-box (SCF) complex, a modular RING-type E3 ubiquitin-ligase, in bovine preimplantation development. It is thought that up to 20% of ubiquitinated proteins are triggered for degradation by the SCF complex [10]. The complex consists of three invariable components—Skp1, Cullin 1 and Rbx1—and one of many F-box proteins, which determines the substrate specificity [11–13]. The deregulation of SCF-complex activity is included in the ethiopathogenesis of many diseases, including cancer [14,15].

Invariant members of the SCF complex

Cullin 1. Cullin 1 is the backbone of the SCF complex and determines its activity. It forms two mutually exclusive complexes whose generation is based on neddylation (modification with the small ubiquitin-like protein Nedd8). When deneddylated, Cullin 1 binds to CAND1 (Cullin-associated and neddylation dissociated 1) and becomes ubiquitination inactive. After neddylation, CAND1 dissociates and Cullin 1 binds to Skp1 and forms the SCFcomplex, which enables protein ubiquitination [16,17].

Cullin 1, as an essential part of this E3 ubiquitin ligase, plays an important role in a large number of biological processes, such as cell cycle regulation, signal transduction, transcription or translation [18–20]. Two detailed studies concerning the role of cullin 1 in early mammalian embryogenesis were published in 1999 [21,22]. However, to the best of our knowledge, no major papers have been published since then. Both Delay and Wang found that Cullin 1 plays a crucial role during embryogenesis before the onset of gastrulation in mice [21,22]. However, its function during preimplantation development has not been specified. In *C. elegans*, cullin 1 likely participates in the degradation of proteins that directs the transformation of an oocyte into a rapidly developing, totipotent embryo [8]. It may play a role in hatching and post-hatching pre-attachment processes, as the expression of its mRNA significantly increases from a blastocyst to a hatched blastocyst [23]. Higher expression was shown to indicate a high quality embryo [24]. Cullin 1 also plays an important role in trophoblast cell invasion and placenta development [25].

We have recently found that cullin 1 is expressed from two different genes, both of them located on chromosome 4, only in distinct regions [26]. One of them was identified as Cullin 1 (NM_001193233) and was expressed from the late 8-cell stage to the blastocyst stage. Because of

its expression, we also call this variant embryonic cullin 1. The other variant shared 83% homology and was expressed from the MII oocyte until the early 8-cell stage. The second variant was called cullin 1-like or maternal cullin 1. In somatic cells, embryonic cullin 1 is expressed.

Skp1 (S-phase kinase-associated protein 1). Skp1 is a stable protein whose best-known functions are connected to the SCF complex [27] Skp1 bounds the catalytic core of the SCF complex to the F-box motif of the F-box protein [11–13]. It participates in the regulation of SCF complex deactivation by the deneddylation of cullin. Altered expression of Skp1 plays a role in the development of Parkinson´s disease [28,29] and lymphomas [30].

Rbx1 (ring-box 1; ROC1—regulator of cullins-1). Rbx1 (together with Cullin 1) forms the catalytic core of the SCF complex. In addition to cullin 1, Rbx1 interacts with all six of the other cullins, and thus also activates other E3 ubiquitin ligases [31,32]. Rbx1 contains the RING finger domain that recruits the E2 ubiquitination enzyme and mediates the neddylation of cullin [33]. Rbx1 plays an unquestionable role in the embryogenesis of *C. elegans*, *D. melanogaster* and even mice, as silencing its mRNA causes embryonic death in these species [34–37]. It is also responsible for the normal progress of mouse and *Xenopus* oocyte meiosis [38–40] Rbx1 RNA silencing causes DNA double strand breaks, G2 arrest and aneuploidy [41], and is overexpressed in many cancers.

Nevertheless, little information is available on the role of the SCF-complex in early embryogenesis. The activity of the SCF complex during mammalian preimplantation development and the expression of each invariant component have not been described to date. In this paper, we describe the expression profile of the mRNA and protein of each component in detail. Furthermore, we define the SCF complex activity from the MII oocyte to blastocyst stage. The preimplantation development of the selected model organism, bovines, is highly similar to humans and other non-rodent mammals in terms of the timing of embryonic genome activation, epigenetic reprogramming, duration of the development and presence of the piRNA and Piwi proteins pool [42]. A similar expression profile can be hence expected in other mammals.

Materials and Methods

IVF and embryo culture

Unless otherwise indicated, the chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) and plastic from Nunclon (Nunc, Roskilde, Denmark). Bovine embryos were obtained after the *in vitro* maturation of oocytes and their subsequent fertilisation and *in vitro* culture. Briefly, abattoir-derived ovaries from cows and heifers were collected and transported in thermocontainers in sterile saline at about 33°C. The cattle had been slaughtered (Jatky Rosovice, spol. s.r.o.; Slaughterhouse Rosovice) for the public edible meat. Those ovaries were discarded without any utilization. Hence, an ethics statement in our paper was not required. The follicles with a diameter between 5 and 9 mm were dissected with fine scissors and then punctured. The cumulus-oocyte complexes were evaluated and selected according to the morphology of the cumulus and subjected to in vitro maturation in TCM 199 (Earle´s salt) supplemented with 20 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% oestrus cow serum (ECS) and gonadotropins (P. G. 600, 15 U/ml; Intervet, Boxmeer, Holland) without a paraffin overlay in four-well dishes under a humidified atmosphere for 24 h at 39°C with 5% CO_2.

For IVF, the cumulus-oocyte complexes were washed four times in PBS and once in Tyrode´s albumin lactate pyruvate (TALP) fertilisation medium, and transferred in groups of up to 30 to four-well dishes containing 250 μl TALP per well. The TALP medium contained 1.5 mg/ml BSA, 30 μg/ml heparin, 0.25 mM sodium pyruvate, 10 mM lactate and 20 μM penicillamine. One straw with frozen semen from one bull previously tested in the IVF system was thawed in a 40°C water bath, diluted with 2 ml TALP and centrifuged at 3500 g for 10 min. The

spermatozoa were layered under 5×1ml TALP. The supernatant with the motile spermatozoa was isolated after 1h of swim-up at 39°C [43]. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of 2×10^6 spermatozoa/ml. A 250 μl aliquot of this suspension was added to each fertilisation well to obtain a final concentration of 1×10^6 spermatozoa/ml. Plates were incubated under a humidified atmosphere with 5% CO_2–5% O_2–90% N_2 for 20 h at 39°C.

At approximately 20 h post fertilisation (hpf), presumed zygotes were denuded by gentle pipetting and transferred to Menezo B2 medium (Veterinary Research Institute, Brno, Czech Republic) supplemented with 10% ECS and cultured in a humidified atmosphere of 5% CO_2–5% O_2–90% N_2 (25 zygotes in 25 μl medium under liquid paraffin (Origio, Malov, Denmark)). The dishes were examined at 24 h post isolation and 34, 44, 72, 96, 120, 156 and 180 hpf, and MII oocytes and two-cell, four-cell, early eight-cell, late eight-cell, morula, blastocysts and hatched blastocysts were collected at each time point respectively.

Quantification of mRNA expression

Poly (A)+ mRNA was extracted from the pools of 20 oocytes and embryos at each stage of development, using a Dynabeads mRNA DIRECT Micro Kit (Invitrogen Dynal AS, Eugene, OR) according to the manufacturer´s instructions. Before isolation, 1 pg of Luciferase mRNA (Promega Madison, WI) per oocyte/embryo was added as an external standard. Primer sequences were designed using Beacon Designer 7 from the bovine cullin 1-like, cullin 1, Skp1 and Rbx1 gene sequences (GeneBank accession numbers XM_589507.3, NM_001193233.1, NM_001034781, NM_001034781) (Table 1).

The expression of specific mRNA was measured by quantitative RT-PCR. mRNA was amplified using a OneStep RT-PCR kit (Qiagen, Hilden, Germany) with real-time detection using SybrGreen fluorescent dye. The reaction composition was Qiagen OneStep RT-PCR buffer (1×), dNTP Mix (400 μM of each), forward and reverse primers (both 400 μM), SybrGreen (1:50 000 of 1000× stock solution; Invitrogen), RNasin Ribonuclease Inhibitor (Promega; 0.2 μl), QIAGEN OneStep Enzyme Mix (0.5 μl), and template RNA. Reaction conditions were as follow: RT at 50°C for 30 min, initial activation at 95°C for 15 min, cycling: denaturation at 94°C for 15 s, annealing at 60°C for 20 s (Cul 1-like, Cul1), at 55°C for 20 s (Skp 1), at 63°C for 20 s (Rbx 1), and extension at 72°C for 30 s. The final extension step was held for 10 min at 72°C. The real-time RT-PCRs were run in duplicate, with all samples (oocytes and all embryos stages) in the same reaction. The experiments were carried out in a RotorGene 3000 (Corbett Research, Morthlake, Australia). Fluorescence data were acquired at 3°C below the melting temperature to distinguish the possible primer dimers.

Table 1. Primer details.

Primer	Sequences	Annealing temperature (°C)	Amplicon size (bp)
Cul 1-like	5´– CGG ACT GGA GCC AGA ATC CCA–3´	60	178
(XM_589507.3)	5´– GTC TGG GCT TGA GGG GAC ACA– 3´		
Cul1	5´– AAC CCC CAC GGA CTC AAG CAG A– 3´	60	173
(NM_001193233.1)	5´– GCC CCT CGA GCT TGG TTT GAC T– 3´		
Skp1	5´– GCC ATC TCC TTG AGC CCT AC– 3´	55	172
(NM_001034781)	5´– CAT TTG GCA AGG GGA CTG GA– 3´		
Rbx1	5´– CAG GCG TCC GCT ACT TCT G– 3´	63	93
(NM_001034781)	5´– TGT TTT GAG CCA GCG AGA GA– 3´		
Luciferase	5´– ACT TCG AAA TGT CCG TTC GG– 3´	55	633
	5´– ACT TCG AAA TGT CCG TTC GG– 3´		

doi:10.1371/journal.pone.0147096.t001

The relative concentration of the template in the different samples was determined using comparative quantification in analysis software (Corbett Research) as described in [44]. The results were normalised according to the relative concentration of the external standard (Luciferase). The take-off points were calculated as 20% of the second-derivative maximum level (RotorGene 3000 operation manual; Corbett Research). Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. Experiments were repeated at least four times.

Alpha-amanitin treatment

To block RNA polymerase II-dependent transcription, α-amanitin (Sigma-Aldrich, St. Louis, MO) was added to the culture medium at a final concentration of 100 μg/ml for either from the one-cell stage to two-cell stage (20–34 hpf), from the one-cell stage to four-cell stage (20–44 hpf), from the four-cell stage to early eight-cell stage (44–72 hpf) or from the four-cell stage to late eight-cell stage (44–96 hpf). After the α-amanitin treatment, the embryos were washed with PBS, immediately frozen and stored at -80°C. Control embryos were collected at the same time interval as their treated counterparts from the same fertilization/cultivation group, washed with PBS, immediately frozen and stored at -80°C. All pools were done in triplicate and contained 20 embryos.

Immunofluorescence

Embryos were fixed in 4% paraformaldehyde for 50 min at 4°C. Fixed embryos were processed immediately or stored in PBS for up to 3 weeks at 4°C. After washing in PBS, the embryos were incubated in 0.5% (v/v) TritonX-100 for 15 min. All subsequent steps were done in PBS supplemented with 0.3% (w/v) BSA and 0.05% (w/v) saponin (PBS/BSA/sap). Embryos were blocked with 2% normal goat serum (NGS; Millipore Biosciences; St. Charles, MO) for 1 h and incubated with rabbit anti-cullin 1 antibody (Abgent AJ 1205a, San Diego, CA) 1:100, rabbit anti- ROC1 (Abcam, Cambridge, UK) 1:100 or mouse anti-SKP 1 (Abcam 4E11, Cambridge, UK) 1:100 in PBS/BSA/sap overnight at 4°C. After thorough washing, the embryos were incubated with goat anti-rabbit antibody conjugated with FITC 1:350 (Santa Cruz Biotechnology, Santa Cruz, TX) or goat anti-mouse conjugated with Alexa Fluor 594 1:800 (Invitrogen, Eugene, OR) in PBS/BSA/sap for 1 h at room temperature in the dark. After washing, the nuclei were stained and the embryos were mounted with Vectashield HardSet Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Controls of immunostaining specificity were carried out by omitting the primary antibody or using another species-specific secondary antibody conjugate.

In situ Proximity ligation assay (PLA)

To test the activity of the SCF complex, the interaction of CUL1 and SKP1 was localized and quantified by Duolink (Olink Bioscience, Uppsala, Sweden). Unless otherwise indicated, chemicals were part of the Duolink. Embryos were fixed and permeabilized in the same way as for immunofluorescence staining. The Duolink in situ proximity ligation assay (PLA) was performed according to the manufacturer´s instructions. Embryos were blocked with 2% normal donkey serum (NDS; Santa Cruz Biotechnology, Santa Cruz, TX) in PBS/BSA/sap. Then they were incubated with rabbit anti-cullin 1 antibody (Abgent AJ 1205a, San Diego, CA) 1:100 together with mouse anti-SKP 1 antibody (Abcam 4E11, Cambridge, UK) 1:100, overnight at 4°C. After thorough washing, the embryos were cultivated with the PLA probes, diluted in the antibody diluent buffer, for 1 h at 37°C. After washing, the ligation solution was added for 30 min at 37°C. After washing in washing buffer A, the amplification stock (5x amplification stock, polymerase, RNase-free water) was added to the embryos for 100 min at 37°C in the

dark. After washing, the nuclei were stained and the embryos were mounted with Vectashield HardSet Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). Controls of the assay were prepared by omitting either the probe or primary antibody.

The samples were examined with a Leica SP 5 (Leica Microsystems AG, Wetzlar, Germany). The images were processed using the software Fiji (http://fiji.sc).

Western blotting

Unless otherwise indicated, chemicals were purchased from Sigma. Embryos and oocytes (25 per extract) were lysed in 15 μl of Blue Loading Buffer (772, Cell Signaling Technology, Danvers, MA) with dithiothreitol, boiled for 5 min and subjected to 12% SDS-PAGE. Proteins were transferred from gels to an Immobilon P membrane (Millipore Biosciences, Billerica, MA) using a semidry blotting system (Whatman Biometra GmbH, Hoettingen, Germany) for 28 min at 5 mA/cm^2. The blocking of the membrane was performed in 3% BSA in TBS-Tween buffer (TBS-T, 20 mM Tris, pH 7.4, 137 mM NaCl and 0.5% Tween 20) for cullin 1 and RBX1, and in 5% non-fat milk in TBS-T for SKP1, for 1 h and incubated overnight with rabbit anti-cullin 1 (Abgent 1205a) 1:1000, rabbit anti-ROC 1 (Abcam) 1:1000 or mouse anti-SKP 1 antibody (Abcam 4E11) 1:1000 in 5% non-fat milk/TBS-T. The results of the SKP 1 antibody were verified with a different mouse anti-SKP1 antibody (Abcam 1H9) 1:1000. After washing in TBS-T, the membranes were incubated with HRP-conjugated donkey anti-rabbit or donkey anti-mouse IgG antibody (both 1:7500; Jackson Immuno Research, Suffolk, UK) in 5% non-fat milk/TBS-T for 1 h at room temperature. Proteins were visualized with Luminata Crescendo Western HRP (Merck Millipore, Darmstadt, Germany). The data were processed using Quantity One software (Bio-Rad).

Statistical analysis

The data were analysed using SigmaStat 3.0 software (Jandel Scientific, San Rafael, CA). The One-Way ANOVA test or Holm-Sidak method were used. $P \leq 0.05$ was considered statistically significant.

Results

The mRNA expression of invariant members of SCF complex

By quantitative RT-PCR analysis with variant-specific primers, both *Cul 1-like* and *Cul1* transcripts were detected in bovine embryos before and after embryonic genome activation (EGA). The expression pattern of the maternal *Cul 1-like* transcript during the in vitro culture (from MII oocyte to blastocyst stage) exhibited a relatively constant high level from MII to 4c, however at the early 8c stage (72 hours post fertilization, hpf), mRNA level rapidly decreased ($p<0,001$) and continued decreasing until the blastocyst stage (Fig 1A). Inversely, the level of *Cul1* mRNA was low from MII to 4c, then rose slightly up to the early 8c (72 hpf), and significantly increased at the late 8c stage (96 hpf) ($p<0.05$). The level was highest at the blastocyst stage (Fig 1B).

The *Skp1* mRNA level was similar to *Cul1* during bovine preimplantation development. The transcript level slightly decreased from MII to 4c and started to rise at early 8c (72 hpf). A significant increase occurred at late 8c ($p<0.05$) and rose further up until the morula stage. Transcript nonsignificantly decreased at the blastocyst stage (Fig 1C).

The *Rbx1* mRNA was stable from MII to 4c, the level fell at early 8c but increased after EGA, at late 8c, and rose rapidly from the morula to blastocyst stage ($p<0.001$) (Fig 1D).

To determine at which stage the transcription of *Cul1* from the embryonic genome begins, embryos were cultured in the presence of α-amanitin, an RNA II polymerase inhibitor. There

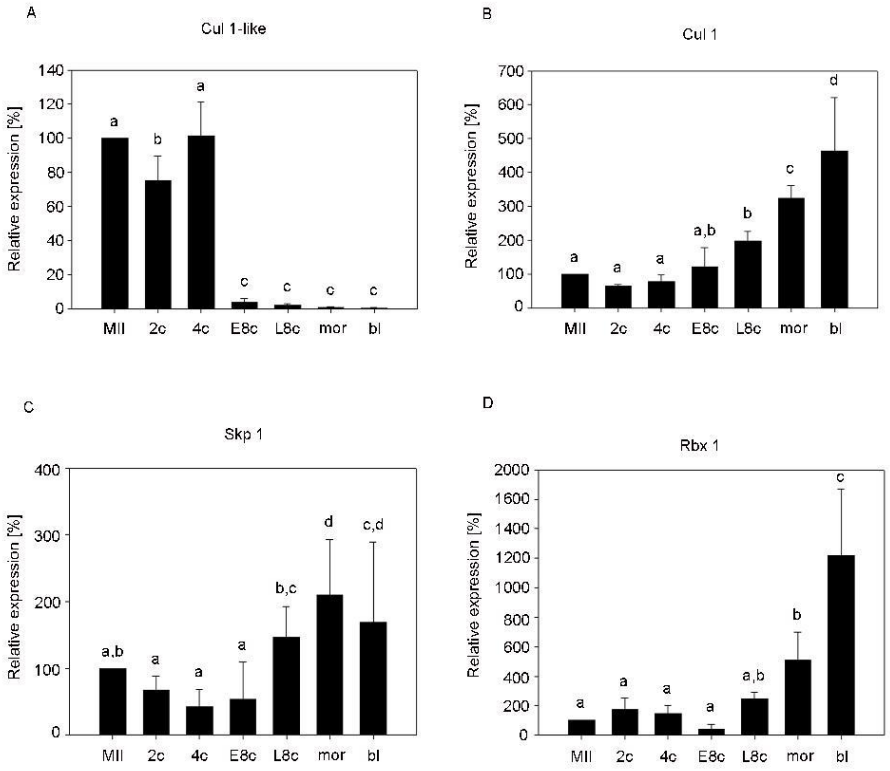


Fig 1. Relative mRNA expression of invariant members of SCF complex. Untreated embryos. The data were normalised according to the relative concentration of the external standard (luciferase mRNA, 1 pg per oocyte/embryo). (A) *Cul 1-like*, (B) *Cul1*, (C) *Skp1*, (D) *Rbx1*. Bars show ± S.D. ^{a,b,c,d}. Values with different superscripts indicate statistical significance (P<0.05). (MII, MII stage oocyte; 2c, two-cell stage embryo; 4c, four-cell stage embryo; E8c, early eight-cell embryo; L8c, late eight-cell stage; mor, morula; bl, blastocyst).

doi:10.1371/journal.pone.0147096.g001

was a significant ($p<0.001$) decrease in *Cul1* and *Skp1* at the 4c and early 8c stage (Fig 2A–2D), respectively, indicating that their transcription is initiated prior to major embryonic genome activation (EGA). On the other hand, the significant difference between the control group and α-amanitin-treated group in *Rbx1* occurred at the late 8c stage (Fig 2E–2F), at the time of the EGA. These results show the importance of the invariant members of the SCF complex in pre-implantation development. The impact of α-amanitin treatment on transcript level at the different stages was also examined (S1 Fig).

Protein expression and localization throughout bovine preimplantation development

Cullin 1. The level of cullin 1 protein gradually increased from MII oocytes (MII) to morula-stage embryos with a significant increase in protein level from the MII to late 8-cell stage and morula stage ($p<0.01$) (Fig 3A).

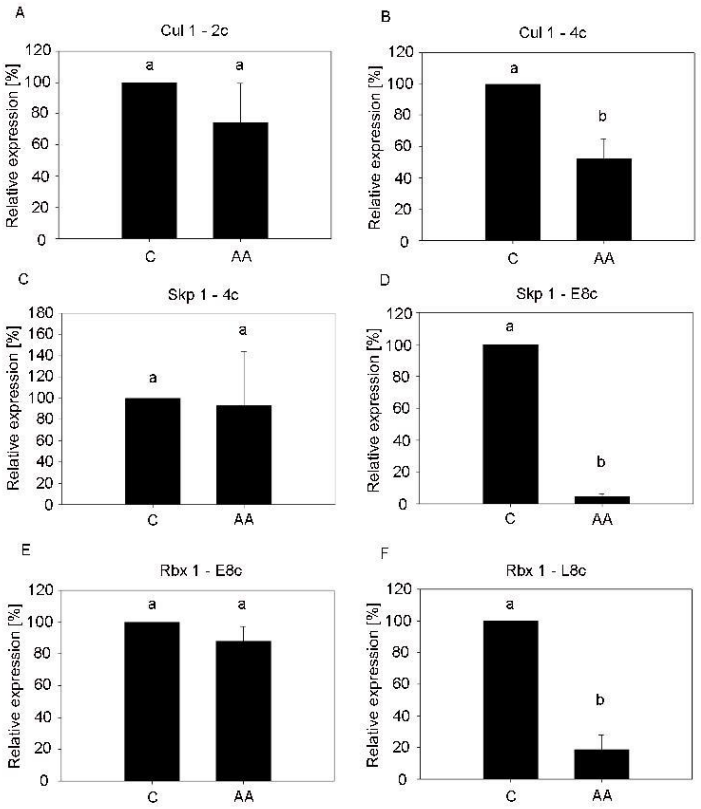


Fig 2. Relative mRNA expression of invariant members of SCF complex, embryos treated with α-amanitin. The data were normalised according to the relative concentration of the external standard (luciferase mRNA, 1pg per embryo). (A) *Cul1* –embryos treated from one-cell to two-cell stage, (B) *Cul1* – embryos treated from one-cell to four-cell stage, (C) *Skp1* –embryos treated from one-cell to four-cell stage, (D) *Skp1* –embryos treated from four-cell to early eight-cell stage, (E) *Rbx1* –embryos treated from four-cell to early eight-cell stage, (F) *Rbx1* –embryos treated from four-cell to late eight-cell stage. Bars show ± S.D. [a,b] Values with different superscripts indicate statistical significance (P<0.05). (C, control group of untreated embryos; AA, group of embryos treated with α-amanitin; 2c, two-cell stage embryo; 4c, four-cell stage embryo; E8c, early eight-stage embryo; L8c, late eight-cell stage embryo).

doi:10.1371/journal.pone.0147096.g002

The localization of Cullin 1 was already described in our previous paper [26], thus here we present only the immunofluorescent staining of blastocysts. The protein was quite abundant throughout the whole embryo and localized primarily to nuclei and to a slightly lesser extent to the cytoplasm. However, we found a difference in the staining of the inner cell mass (ICM) and trophectoderm (TE). Even though Cullin 1 was present in both of them, the staining was noticeably less intensive in the ICM (Fig 4).

SKP1. The level of SKP1 protein significantly increased from MII oocytes to 4c embryos, but then significantly decreased again ($p<0.05$ in all cases). There were no statistically

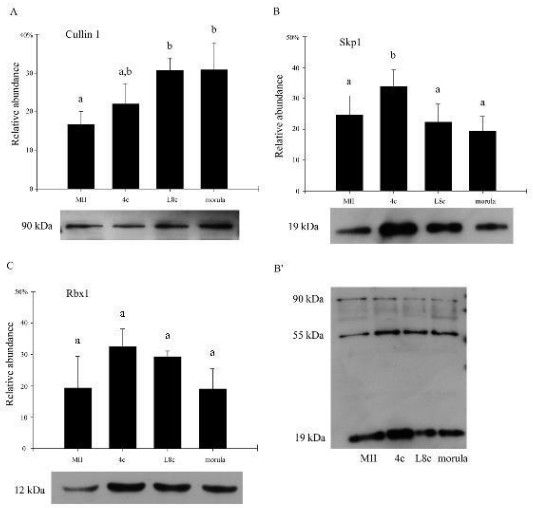


Fig 3. Quantification of protein level after western blot analysis of bovine oocytes and preimplantation embryos. 30 embryos per lane. A) Cullin 1; B) SKP1 (antibody 4E11); C) RBX1. The data were processed using Quantity One software (Bio-Rad). 100% represents the sum of the trace quantities of all bands; relative abundance (y-axis) represents the percentage of each band. Bars show mean ± S.D. [a,] [b]Values with different superscripts indicate statistical significance (P<0.05). The experiments were repeated at least three times, and representative western blot images are shown below the graph. B') Representative image of additional bands (approximate size 55 and 90 kDa). (MII, MII oocytes; L8c, late eight-cell stage embryos; 4c, four-cell stage embryos).

doi:10.1371/journal.pone.0147096.g003

significant differences between MII oocytes, L8c embryos and morulas ($p>0.05$) (Fig 3B). In addition to the expected cca 19-kDa band, we detected two other weaker bands with approximate sizes of 55 and 90 kDa (Fig 3B'). This finding was verified using another SKP1-specific antibody (S2 Fig).

The protein was distributed throughout the cell, and to some extent was also present at the zona pellucida. In MII oocytes, we found an accumulation of the immunofluorescence signal around the nucleus. In blastocysts, the staining was slightly less intensive in the ICM (Fig 5).

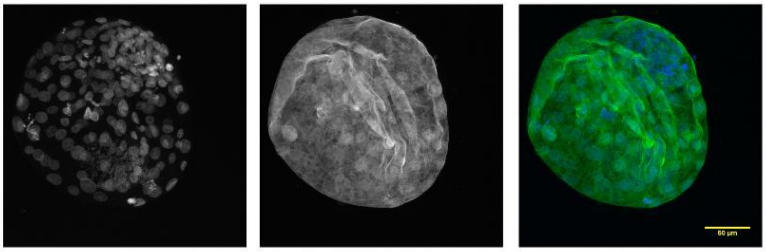


Fig 4. Confocal laser scanning microscopy of CUL1 of bovine blastocysts. Nuclei (DAPI)–blue; cullin 1 –green.

doi:10.1371/journal.pone.0147096.g004

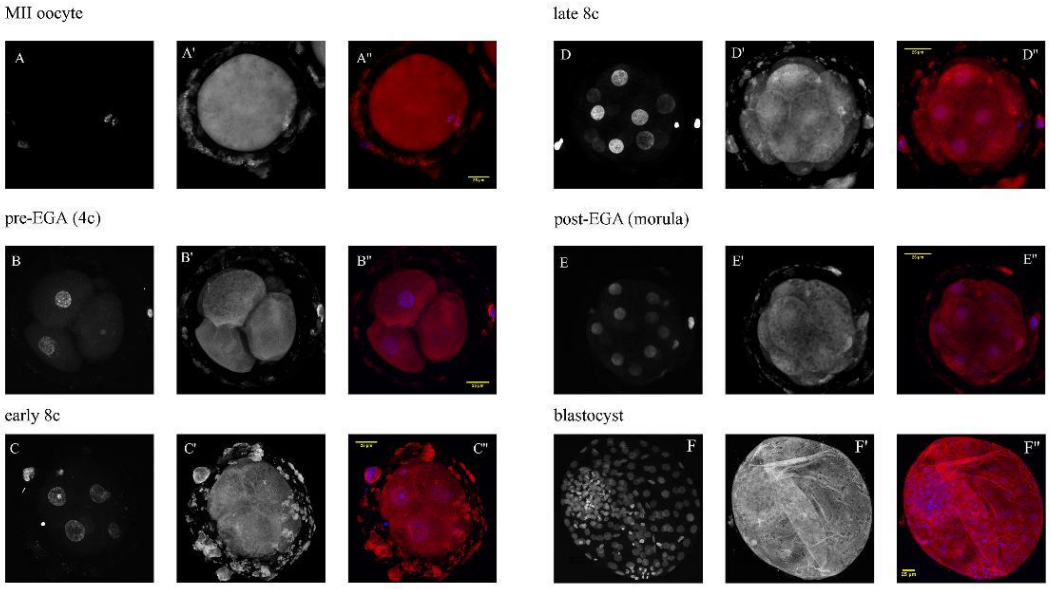


Fig 5. Confocal laser scanning microscopy of SKP1 from MII oocytes to blastocyst-stage embryos. The embryos were labelled with mouse monoclonal anti-SKP1 antibody (A'–F') and the nuclei were stained with DAPI (A—F). In overlaid images (A"–F"), SKP1 is red and DNA blue.

doi:10.1371/journal.pone.0147096.g005

RBX1. There were no statistically significant differences between the developmental stages ($p>0.05$) (Fig 3C). However, in MII oocytes and morulas, the protein was noticeably less abundant than in 4c and L8c. The protein was localized to the nucleus and cytoplasm at all developmental stages. At the blastocyst stage, the protein seems to be localized to the ICM and TE with the same intensity (Fig 6).

SCF complex activity

The interaction of Cullin 1 and Skp1, which indicates SCF complex activity, was monitored in groups consisting of MII oocytes, 4c embryos, late 8c embryos and morulas (Fig 7A–7D´´). The intensity of the fluorescence signal was measured and compared between these developmental stages. Blastocysts were analysed in a separate experiment. There was no statistically significant difference in PLA signal intensity between the groups; however, in MII oocytes the signal was noticeably more intensive than in all embryonic stages (Fig 8). At the blastocyst stage, there was almost no PLA signal in the ICM and on the other hand, the signal was very intensive in the TE (Fig 7E–7E´´).

Discussion

The function of the SCF complex and its members in somatic cells, its necessity for oogenesis [39,45–48] spermatogenesis [49,50] and early development [8] and the need for precise regulation of proteolytic processes in early embryogenesis suggest the potential importance of the complex during mammalian preimplantation development.

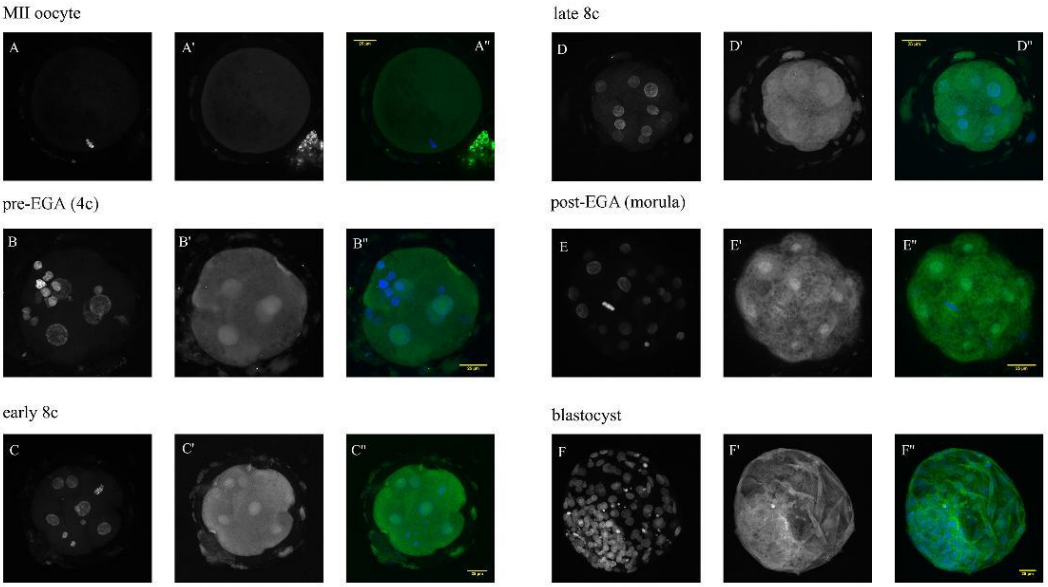


Fig 6. Confocal laser scanning microscopy of RBX1 from MII oocytes to blastocyst-stage embryos. The embryos were labelled with rabbit monoclonal anti-RBX1 antibody (A'—F') and the nuclei were stained with DAPI (A–F). In overlaid images (A"–F"), RBX1 is green and DNA blue.

doi:10.1371/journal.pone.0147096.g006

To the best of our knowledge, the role of the SCF complex during this period has not been examined to date, nor has the expression profile of its members been determined. However, it is not possible to study the involvement of the SCF complex in preimplantation protein degradation without this knowledge. Thus, we determined thorough expression profiles of both the mRNAs and proteins of three invariant SCF complex members (Cullin 1, Rbx1, and Skp1). We also described the course of SCF complex activity throughout preimplantation development.

The dynamics of mRNA expression between individual stages reflects the importance of the gene during preimplantation development. Genes activated no later than during the major wave of EGA are assumed to be the most important. It was shown that silencing genes activated before or during EGA often results in early developmental arrest or substantially lowers its quality [51–53]. Genes related to protein ubiquitination are usually activated at the 8-cell stage [54]. However, we found earlier activation of two of the three invariant members of the SCF complex. The embryonic transcription of *Cul1* and *Skp1* starts in the 4c and early 8c stage, respectively, the transcription of *Rbx1* during a major wave of EGA (Fig 1). This is why the necessity of these genes and in fact the whole of the SCF complex for the normal course of EGA seems to be undisputed. The expression of Cullin 1 starts in the very early stages of embryogenesis, both in bovines and Drosophila [55]. Thus the embryonic expression of Cullin 1 seems to be the most important of the SCF complex members. This is further supported by the existence of the two Cullin 1 variants. These transcripts represent the products of two different genes, both present on chromosome 4 (UniGene IDs: maternal cullin 1—BT.36789; embryonic cullin 1—BT.6490) [26]. The transcripts share 83% identity, however the predicted protein of maternal cullin 1 shares only 78% identity with GenPept protein ID:

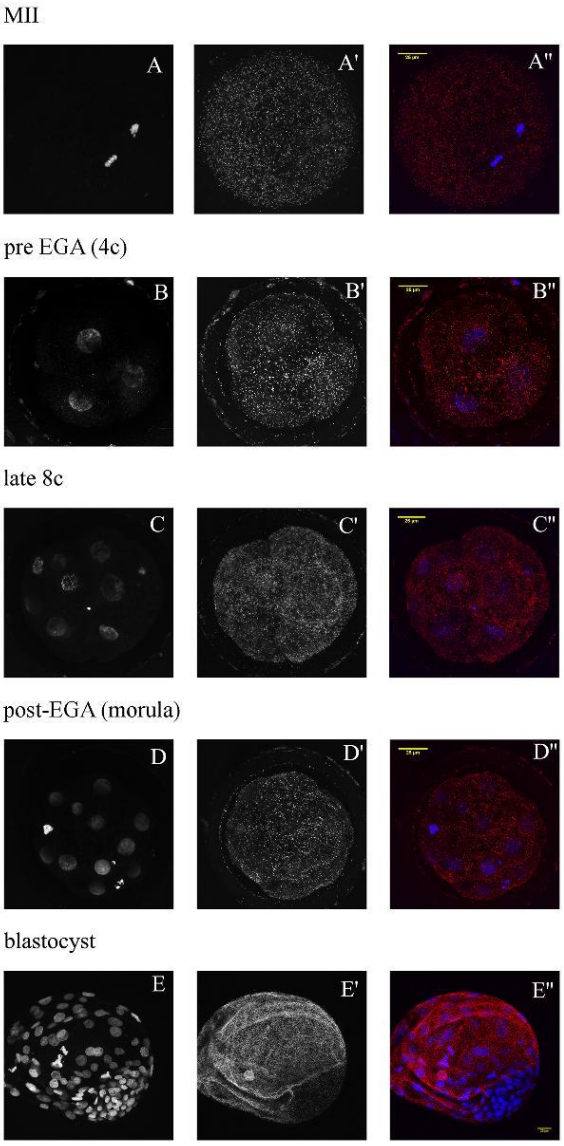


Fig 7. Confocal laser scanning microscopy of MII oocytes and preimplantation embryos after Duolink *in situ* PLA analysis. PLA signal indicates Cul1-Skp1 interaction (A'-E') and the nuclei were stained with DAPI (A-E). In merges (A"-E"), PLA signal is red and DNA blue.

doi:10.1371/journal.pone.0147096.g007

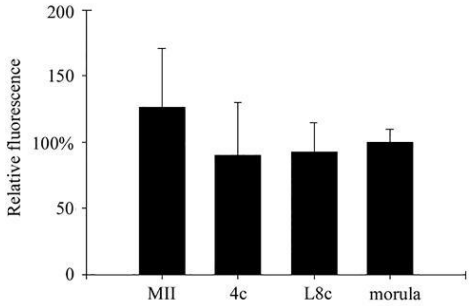


Fig 8. Relative fluorescence after analysis of Skp1/Cul1 interaction. The relative fluorescence (y-axis) represents the emitted fluorescence signal in a single embryo normalised to the mean of the measured fluorescence signal of morulas. There were in total 15 embryos per experimental group. The embryos were analysed using confocal scanning microscope in groups. Each group included an MII oocyte, a 4-cell stage embryo, late 8-cell stage embryo and morula. (MII, MII oocytes; L8c, late eight-cell stage embryos; 4c, four-cell stage embryos).

doi:10.1371/journal.pone.0147096.g008

NP_001180162.1 –cullin 1, *Bos taurus*. The maternal variant is probably a pseudogene raised by gene duplication [26] and is referred to as the *Bos taurus* cullin 1 pseudogene (LOC539792). Filippov [55] found that in *Drosophila* only the protein, not the transcript, is maternally inherited from the oocyte to embryo. This could be actually caused by the existence of two mRNA variants and non-detection of the maternal one. A similar expression change during preimplantation development was found in murine eIF-1A. In this case, the maternal and embryonic transcripts utilize different promoters, giving rise to diverse transcriptional products. In oocytes a TATA-containing promoter is used, while in embryos a TATA-less promoter is used [56]. The authors hypothesize that the increased utilization of the TATA-less promoter during EGA and further embryonic development can contribute to the smooth running of gene expression reprogramming, and is related to an increased expression of housekeeping genes or totipotent marker Oct4, which are both expressed from a TATA-less promoter. However both Cul1 variants are expressed from a TATA-less promoter, and thus the expression shift cannot be associated with different promoter utilization. Another similar case is the expression of the pseudogenes Zscan4d and Zscan4c in 2-cell stage murine embryos and murine embryonic stem cells, respectively [52]. However, in this case Zscan4 pseudogenes are not expressed in any other tissues or cell types, or maternally in the oocyte. Thus to the best of our knowledge, such a shift as in Cullin 1 variant expression has not been described elsewhere to date. Yet it seems that the activation of embryonic Cullin 1 transcription is important for normal preimplantation development, since it takes place as early as in the 4c stage. Even though at this stage the maternal variant was still predominant, almost no maternal cullin 1 was detected at the early 8c stage, and we can say that from this stage onwards the embryo is fully dependent on the embryonic variant.

Using Duolink in-situ PLA analysis, we monitored the interaction of Cullin 1 and Skp1, which indicates the activity of the SCF complex. The PLA signal was found in all developmental stages and was comparably intense in the examined embryonic stages. The highest activity was found in MII oocytes, where the proper degradation of protein is crucially important for normal maturation, however even this difference was not statistically significant. Generally, APC/C is thought to be the most important ubiquitin-ligase for meiotic maturation reviewed in [57], but it was found that the degradation of mitotic APC/C is controlled by the SCF

complex [50]. Hence we hypothesize that the SCF complex also plays a similar role during meiosis. The PLA results indicates that the SCF complex is likely required for controlling the whole of preimplantation development, and that both maternal and embryonic variants of cullin 1 should be functional. However, since embryonic cullin 1 mRNA starts to be transcribed as early as the 4c stage, the shift seems to be required for preparing the embryo for EGA and it is still needed in further stages of preimplantation development. The level of cullin 1 protein gradually increased from MII oocytes (MII) to the morula stage. Since the localization of cullin 1 protein in bovine preimplantation embryos has been described in our previous paper [26], here we only present the data obtained in blastocysts. From the 2c to morula stage, the protein is dispersed throughout the blastomere and is present in the whole embryo [26]. Here we have found that at the blastocyst stage, cullin 1 tends to also localise to nuclei and it started to be predominantly localized in the trophectoderm rather than the inner cell mass. This is in accordance with the fact that Cullin 1 plays a key role in trophoblast cell invasion and placenta development [24]. An even greater difference was found in the staining intensity between TE and ICM after PLA staining. Almost no signal was detected in the ICM. These findings confirm the occurrence of ubiquitination in the trophectoderm [58]. Surprisingly, this activity did not correlate with the protein abundance of any of the three examined genes. Cullin 1 is thought to be the limiting factor for SCF complex activity [59], however the protein level increased gradually from MII oocytes to L8c-stage embryos, and then remained stable at the morula stage. The PLA activity was the highest in MII oocytes, nonsignificantly, though noticeably decreased in the 4c stage and remained stable until the morula stage. Thus both the localization of the proteins and the PLA signal at the blastocyst stage and the progress of protein level and SCF complex activity suggest that all three basic components of the SCF complex, including Cullin 1, also have other functions than merely being components of the SCF complex. This is especially obvious in Rbx1, which is known to interact with all other members of the cullin family, and thus is also part of other E3 ligases [30,60]. Both the mRNA and protein expression of Rbx1 defy the expression profiles of the other two invariant members of the SCF complex. Thus, even though Rbx1 is necessary for SCF complex functionality, and the complex is non-functional after Rbx1 silencing [40], RBX1 expression does not correlate with SCF complex activity. This applies to a lesser extent for the other two proteins, even though to the best of our knowledge all currently known functions of cullin 1 are related to the SCF complex, and Skp1 has a few functions besides the SCF complex in lower organisms [27,61]. We assume that the presumptive Skp1 complexes represented by the higher bands after western blot analysis are also not related to the SCF complex because of their size.

In conclusion, the mRNA and protein expression of the basic components of the SCF complex (Cullin 1, Skp1 and Rbx1) suggest that all these genes are essential for normal preimplantation development. The early activation of *Cul1* and *Skp1* mRNA expression suggests that these genes are necessary for preparing the embryo for EGA. SCF complex-mediated ubiquitination takes place at approximately the same level throughout the whole of preimplantation development. However at the blastocyst stage, the ubiquitination is concentrated specifically to the trophectoderm, the emerging embryonic part of the placenta. The comparison of protein expression and SCF complex activity showed that protein abundance does not correlate with the complex activity. At the MII stage, proteins were expressed at a low level, but the SCF complex activity was the highest. However, this does not mean that the level of protein expression does not influence ubiquitination by the SCF complex, as was shown by Piva and coauthors [62]. Altogether, our data suggest that SCF complex mediated protein degradation plays an important role in EGA initiation in bovines.

Supporting Information

S1 Fig. Relative mRNA expression of invariant members of SCF complex, embryos treated with α-amanitin, supplement. The data were normalised according to the relative concentration of the external standard (luciferase mRNA, 1pg per embryo). (A) *Cul1*, (B) *Skp1*, (C) *Rbx1*. Bars show ± S.D. [a,b] Values with different superscripts indicate statistical significance ($P<0.05$). (C, control group of untreated embryos; AA, group of embryos treated with α-amanitin; 2c, two-cell stage embryo; 4c, four-cell stage embryo; E8c, early eight-stage embryo; L8c, late eight-cell stage embryo).
(TIFF)

S2 Fig. Western blot analysis of bovine oocytes and preimplantation embryos using anti-SKP1 antibody 1H9. 30 embryos per lane. A) Quantification of protein level. The data were processed using Quantity One software (Bio-Rad). 100% represents the sum of the trace quantities of all bands; relative abundance (y-axis) represents the percentage of each band. Bars show mean ± S.D. [a,b]Values with different superscripts indicate statistical significance ($P<0.05$). The experiment was repeated four times, and a representative western blot image is shown below the graph. B) Representative image of additional bands (approximate size 55 and 90 kDa). (MII, MII oocytes; L8c, late eight-cell-stage embryos; 4c, four-cell-stage embryos).
(TIFF)

S3 Fig. Confocal laser scanning microscopy of SKP1 (antibody 1H9) from MII oocytes to blastocyst-stage embryos. The embryos were labelled with mouse monoclonal anti-SKP1 antibody 1H9 (A'—F') and the nuclei were stained with DAPI (A–F). In overlaid images (A"–F"), SKP1 is red and DNA blue.
(TIF)

S4 Fig. Western blot analysis of SKP1 using Abnova antibody. First band shows SKP1 on zona pellucida, second band shows SKP1 in embryos.
(TIF)

Acknowledgments

The authors are indebted to R. Sucha and A. Susor for their helpful comments and expert assistance during the experiments; to M. Kopcikova, J. Kankova, J. Supolikova and M. Rakocyova for their excellent technical assistance.

Author Contributions

Conceived and designed the experiments: VB JK TT. Performed the experiments: VB VK JK TT. Analyzed the data: VB TT. Contributed reagents/materials/analysis tools: VB JK TT. Wrote the paper: VB TT.

References

1. Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori KJ, et al. Non-radioisotopic quantitative RT-PCR to detect changes in mRNA levels during early mouse embryo development. Biochem Biophys Res Commun. 1993; 195:769–75. PMID: 7690561
2. Svarcova O, Laurincik J, Avery B, Mlyncek M, Niemann H, Maddox-Hyttel P. Nucleolar development and allocation of key nucleolar proteins require de novo transcription in bovine embryos. Mol Reprod Dev. 2007; 74:1428–35. PMID: 17410544
3. Ohsugi M, Zheng P, Baibakov B, Li L, Dean J. Maternally Derived FILIA-MATER Complex Localizes Asymmetrically in Cleavage-Stage Mouse Embryos. Development. 2008; 135:259–69. PMID: 18057100

4. Toralová T, Benešová V, Kepková KV, Vodička P, Šušor A, Ka ka J. Bovine preimplantation embryos with silenced nucleophosmin mRNA are able to develop until the blastocyst stage. Reprod Camb Engl. 2012; 144:349–59.
5. Suzumori N, Burns KH, Yan W, Matzuk MM. RFPL4 interacts with oocyte proteins of the ubiquitin-proteasome degradation pathway. Proc Natl Acad Sci U S A. 2003; 100:550–5. PMID: 12525704
6. DeRenzo C, Seydoux G. A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends Cell Biol. 2004; 14:420–6. PMID: 15308208
7. Mtango NR, Latham KE. Ubiquitin proteasome pathway gene expression varies in rhesus monkey oocytes and embryos of different developmental potential. Physiol Genomics. 2007; 31:1–14. PMID: 17550997
8. Verlhac M-H, Terret M-E, Pintard L. Control of the oocyte-to-embryo transition by the ubiquitin-proteolytic system in mouse and C. elegans. Curr Opin Cell Biol. 2010; 22:758–63. doi: 10.1016/j.ceb.2010.09.003 PMID: 20943362
9. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev. 2002; 82:373–428. PMID: 11917093
10. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature. 2009; 458:732–6. doi: 10.1038/nature07884 PMID: 19360080
11. Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, et al. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell.1996; 86:263–74. PMID: 8706131
12. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell. 1997; 91:209–19. PMID: 9346238
13. Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, et al. CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. Mol Cell. 2002; 10:1519–26. PMID: 12504026
14. Latres E, Chiarle R, Schulman BA, Pavletich NP, Pellicer A, Inghirami G, et al. Role of the F-box protein Skp2 in lymphomagenesis. Proc Natl Acad Sci U S A. 2001; 98:2515–20. PMID: 11226270
15. Li H-H, Kedar V, Zhang C, McDonough H, Arya R, Wang D-Z, et al. Atrogin-1/muscle atrophy F-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an SCF ubiquitin ligase complex. J Clin Invest. 2004; 11:1058–71.
16. Liu J, Furukawa M, Matsumoto T, Xiong Y. NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. Mol Cell. 2002; 10:1511–8. PMID: 12504025
17. Zheng N, Schulman BA, Song L, Miller JJ, Jeffrey PD, Wang P, et al. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature. 2002; 416:703–9. PMID: 11961546
18. Pennetier S, Uzbekova S, Guyader-Joly C, Humblot P, Mermillod P, Dalbiès-Tran R. Genes preferentially expressed in bovine oocytes revealed by subtractive and suppressive hybridization. Biol Reprod. 2005; 73:713–20. PMID: 15930321
19. Skaar JR, Pagano M. Control of cell growth by the SCF and APC/C ubiquitin ligases. Curr Opin Cell Biol. 2009; 21:816–24. doi: 10.1016/j.ceb.2009.08.004 PMID: 19775879
20. Silverman JS, Skaar JR, Pagano M. SCF ubiquitin ligases in the maintenance of genome stability. Trends Biochem Sci. 2012; 37:66–73. doi: 10.1016/j.tibs.2011.10.004 PMID: 22099186
21. Dealy MJ, Nguyen KV, Lo J, Gstaiger M, Krek W, Elson D, et al. Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. Nat Genet. 1999; 23:245–8. PMID: 10508527
22. Wang Y, Penfold S, Tang X, Hattori N, Riley P, Harper JW, et al. Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. Curr Biol CB. 1999; 9:1191–4. PMID: 10531039
23. Hwang K-C, Cui X-S, Park S-P, Shin M-R, Park S-Y, Kim E-Y, et al. Identification of differentially regulated genes in bovine blastocysts using an annealing control primer system. Mol Reprod Dev. 2004; 69:43–51. PMID: 15278903
24. Lee YL, Lee KF, Xu JS, Kwok KL, Luk JM, Lee WM, et al. Embryotrophic factor-3 from human oviductal cells affects the messenger RNA expression of mouse blastocyst. Biol Reprod. 2003; 68:375–82. PMID: 12533399
25. Zhang Q, Chen Q, Lu X, Zhou Z, Zhang H, Lin H-Y, et al. CUL1 promotes trophoblast cell invasion at the maternal-fetal interface. Cell Death Dis. 2013; 4:e502. doi: 10.1038/cddis.2013.1 PMID: 23429288
26. Kepkova KV, Vodicka P, Toralova T, Lopatarova M, Cech S, Dolezel R, et al. Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression

during maternal-to-embryonic transition. Theriogenology. 2011; 75:1582–95. doi: 10.1016/j.theriogenology.2010.12.019 PMID: 21411133

27. Galan JM, Wiederkehr A, Seol JH, Haguenauer-Tsapis R, Deshaies RJ, Riezman H, et al. Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Mol Cell Biol. 2001; 21:3105–17. PMID: 11287615
28. Mandel SA, Fishman-Jacob T, Youdim MBH. Targeting SKP1, an ubiquitin E3 ligase component found decreased in sporadic Parkinson's disease. Neurodegener Dis. 2012; 10:220–3. doi: 10.1159/000333223 PMID: 22205206
29. Rhodes SL, Fitzmaurice AG, Cockburn M, Bronstein JM, Sinsheimer JS, Ritz B. Pesticides that inhibit the ubiquitin-proteasome system: effect measure modification by genetic variation in SKP1 in Parkinson's disease. Environ Res. 2013; 126:1–8. doi: 10.1016/j.envres.2013.08.001 PMID: 23988235
30. Piva R, Liu J, Chiarle R, Podda A, Pagano M, Inghirami G. In vivo interference with Skp1 function leads to genetic instability and neoplastic transformation. Mol Cell Biol. 2002; 22:8375–87. PMID: 12417738
31. Ohta T, Michel JJ, Xiong Y. Association with cullin partners protects ROC proteins from proteasome-dependent degradation. Oncogene. 1999; 18:6758–66. PMID: 10597284
32. Ohta T, Michel JJ, Schottelius AJ, Xiong Y. ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. Mol Cell. 1999; 3:535–41. PMID: 10230407
33. Morimoto M, Nishida T, Nagayama Y, Yasuda H. Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. Biochem Biophys Res Commun. 2003; 301:392–8. PMID: 12565873
34. Sasagawa Y, Urano T, Kohara Y, Takahashi H, Higashitani A. Caenorhabditis elegans RBX1 is essential for meiosis, mitotic chromosomal condensation and segregation, and cytokinesis. Genes Cells Devoted Mol Cell Mech. 2003; 8:857–72.
35. Moore R, Boyd L. Analysis of RING finger genes required for embryogenesis in C. elegans. Genes N Y N 2000. 2004; 38:1–12.
36. Tan M, Davis SW, Saunders TL, Zhu Y, Sun Y. RBX1/ROC1 disruption results in early embryonic lethality due to proliferation failure, partially rescued by simultaneous loss of p27. Proc Natl Acad Sci U S A. 2009; 106:6203–8. doi: 10.1073/pnas.0812425106 PMID: 19325126
37. Jia L, Bickel JS, Wu J, Morgan MA, Li H, Yang J, et al. RBX1 (RING box protein 1) E3 ubiquitin ligase is required for genomic integrity by modulating DNA replication licensing proteins. J Biol Chem. 2011; 286:3379–86. doi: 10.1074/jbc.M110.188425 PMID: 21115485
38. Gutierrez GJ, Vögtlin A, Castro A, Ferby I, Salvagiotto G, Ronai Z, et al. Meiotic regulation of the CDK activator RINGO/Speedy by ubiquitin-proteasome-mediated processing and degradation. Nat Cell Biol. 2006; 8:1084–94. PMID: 16964245
39. Marangos P, Verschuren EW, Chen R, Jackson PK, Carroll J. Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1). J Cell Biol. 2007; 176:65–75. PMID: 17190794
40. Zhou L, Yang Y, Zhang J, Guo X, Bi Y, Li X, et al. The role of RING box protein 1 in mouse oocyte meiotic maturation. PloS One. 2013; 8:e68964. doi: 10.1371/journal.pone.0068964 PMID: 23874827
41. Jia L, Soengas MS, Sun Y. ROC1/RBX1 E3 ubiquitin ligase silencing suppresses tumor cell growth via sequential induction of G2-M arrest, apoptosis, and senescence. Cancer Res. 2009; 69:4974–82. doi: 10.1158/0008-5472.CAN-08-4671 PMID: 19509229
42. Roovers EF, Rosenkranz D, Mahdipour M, Han C-T, He N, Chuva de Sousa Lopes SM, et al. Piwi proteins and piRNAs in mammalian oocytes and early embryos. Cell Rep. 2015; 10:2069–82. doi: 10.1016/j.celrep.2015.02.062 PMID: 25818294
43. Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev. 1992; 31:63–7. PMID: 1562328
44. Kanka J, Kepková K, Nemcová L. Gene expression during minor genome activation in preimplantation bovine development. Theriogenology. 2009; 72(4):572–83. doi: 10.1016/j.theriogenology.2009.04.014 PMID: 19501393
45. Muzzopappa M, Wappner P. Multiple roles of the F-box protein Slimb in Drosophila egg chamber development. Dev Camb Engl. 2005; 132:2561–71.
46. Doronkin S, Djagaeva I, Beckendorf SK. The COP9 signalosome promotes degradation of Cyclin E during early Drosophila oogenesis. Dev Cell. 2003; 4:699–710. PMID: 12737805
47. Ohlmeyer JT, Schüpbach T. Encore facilitates SCF-Ubiquitin-proteasome-dependent proteolysis during Drosophila oogenesis. Dev Camb Engl. 2003; 130:6339–49.

48. Shcherbata HR, Althauser C, Findley SD, Ruohola-Baker H. The mitotic-to-endocycle switch in Drosophila follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. Dev Camb Engl. 2004; 131:3169–81.

49. Bader M, Arama E, Steller H. A novel F-box protein is required for caspase activation during cellular remodeling in Drosophila. Dev Camb Engl. 2010; 137:1679–88.

50. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, et al. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. Dev Cell. 2003; 4:799–812. PMID: 12791266

51. Toralová T, Susor A, Nemcová L, Kepková K, Kanka J. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. Reprod Camb Engl. 2009; 138:783–91.

52. Falco G, Lee S-L, Stanghellini I, Bassey UC, Hamatani T, Ko MSH. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Dev Biol. 2007; 307:539–50. PMID: 17553482

53. Salilew-Wondim D, Hölker M, Rings F, Phatsara C, Mohammadi-Sangcheshmeh A, Tholen E, et al. Depletion of BIRC6 leads to retarded bovine early embryonic development and blastocyst formation in vitro. Reprod Fertil Dev. 2010; 22:564–79. doi: 10.1071/RD09112 PMID: 20188030

54. Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H, Wolf E. Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments. Anim Reprod Sci. 2014; 149:46–58. doi: 10.1016/j.anireprosci.2014.05.016 PMID: 24975847

55. Filippov V, Filippova M, Sehnal F, Gill SS. Temporal and spatial expression of the cell-cycle regulator cul-1 in Drosophila and its stimulation by radiation-induced apoptosis. J Exp Biol. 2000; 203:2747–56. PMID: 10952875

56. Davis W, Schultz RM. Developmental change in TATA-box utilization during preimplantation mouse development. Dev Biol. 2000; 218:275–83. PMID: 10656769

57. Karabinova P, Kubelka M, Susor A. Proteasomal degradation of ubiquitinated proteins in oocyte meiosis and fertilization in mammals. Cell Tissue Res. 2011; 346:1–9. doi: 10.1007/s00441-011-1235-1 PMID: 21969023

58. Sutovsky P, Motlik J, Neuber E, Pavlok A, Schatten G, Palecek J, et al. Accumulation of the proteolytic marker peptide ubiquitin in the trophoblast of mammalian blastocysts. Cloning Stem Cells. 2001; 3:157–61. PMID: 11945225

59. Liu J, Furukawa M, Matsumoto T, Xiong Y. NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. Mol Cell. 2002; 10:1511–8. PMID: 12504025

60. Zhang H, Kobayashi R, Galaktionov K, Beach D. p19skp1 and p45skp2 are essential elements of the cyclin A-CDK2 S phase kinase. Cell. 1995; 82:915–25. PMID: 7553852

61. Kaplan KB, Hyman AA, Sorger PK. Regulating the yeast kinetochore by ubiquitin-dependent degradation and Skp1p-mediated phosphorylation. Cell. 1997; 91:491–500. PMID: 9390558

62. Piva R, Liu J, Chiarle R, Podda A, Pagano M, Inghirami G. In vivo interference with Skp1 function leads to genetic instability and neoplastic transformation. Mol Cell Biol. 2002; 22:8375–87. PMID: 12417738

Chapter 17

Potential Involvement of SCF-Complex in Zygotic Genome Activation During Early Bovine Embryo Development

Veronika Benesova, Veronika Kinterova, Jiri Kanka, and Tereza Toralova

Abstract

Proper timing of degradation of maternal protein reserves is important for early embryonic development. The major modification that triggers proteins to degradation is ubiquitination, mediated by ubiquitin-proteolytic system. We focus here on Skp 1-Cul 1-F-box complex (SCF-complex), E3 ubiquitin-ligase, a part of ubiquitin-proteolytic system, which transfer ubiquitin to the substrate protein. We describe in this chapter the methods for the characterization of the expression profile of mRNA and protein of invariant members of SCF-complex and for the definition of SCF-complex activity.

Key words SCF-complex, Preimplantation development, qRT-PCR, Western blot, Immunofluorescence, In situ proximity ligation assay

1 Introduction

Early embryo development is driven by maternal mRNAs and proteins. However, during the development these reserves are gradually degraded and replaced with mRNAs produced by newly activated embryonic genome. The degradation of mRNAs peaks around embryonic genome activation and results in the degradation of the vast majority of maternal mRNAs [1]. However, little information about the protein degradation is available. It is thought that a large proportion of the degradation of maternal proteins is mediated by the ubiquitin-proteolytic system [2, 3]. Ubiquitination of proteins is managed by the cooperation of three enzyme complexes: E1—ubiquitin activating enzyme, E2—ubiquitin conjugating enzyme, and E3—ubiquitin ligase [4].

Previously, we characterized the SCF-complex (Skp 1-Cullin 1-F-box), a modular RING type E3 ubiquitin-ligase, in bovine preimplantation embryos [5]. This complex consists of three invariable components—Skp1, Cullin 1 (Cul1) and Rbx1, and one of many F-box proteins, which determines the substrate specificity

Kiho Lee (ed.), *Zygotic Genome Activation: Methods and Protocols*, Methods in Molecular Biology, vol. 1605,
DOI 10.1007/978-1-4939-6988-3_17,

[6–8]. It is thought that up to 20% of ubiquitinated proteins are triggered for degradation by the SCF-complex [9].

Technologies and methods used to characterize the expression profile of SCF-complex in bovine embryos are presented below. The profile of the mRNA was measured by the quantitative RT-PCR. To determine at which stage the transcription of each mRNA from the embryonic genome begins, the embryos were cultured with α-amanitin, specific inhibitor of RNA polymerase II. For the expression profile and localization of proteins, the western blot and immunofluorescence were used. To define the SCF-complex activity during bovine preimplantation development in situ proximity ligation assay was used. This method enables detection of the protein-protein interaction and moreover the localization of this interaction.

2 Materials

2.1 Oocytes and Embryos Preparation

Abattoir-derived ovaries from cows and heifers were collected and transported in thermocontainers in sterile saline at about 33 °C.

2.2 α-Amanitin Treatment

This treatment is used to block the RNA polymerase II-dependent transcription.

1. Add α-amanitin (Sigma-Aldrich, St. Louis, MO) to the culture medium at a final concentration of 100 μg/ml for either from the 1-cell stage to 2-cell stage (20–34 hpf), from the 1-cell stage to 4-cell stage (20–44 hpf), from the 4-cell stage to early 8-cell stage (44–72 hpf) or from the 4-cell stage to late 8-cell stage (44–96 hpf).
2. Collect the embryos at required stage. Collect the control embryos (without added α-amanitin) at the same time interval.
3. Wash embryos in PBS.
4. Place it on ice or store at −80 °C.
5. Use the embryos for Subheading 3.3.

2.3 Buffers

1. PBS/BSA/sap: phosphate-buffered saline (PBS) supplemented with 0.3% (w/v) BSA and 0.05% (w/v) saponin.
2. TBS-Tween: 20 mM Tris, pH 7.4, 137 mM NaCl, and 0.5% Tween 20.
3. 10% Towbin: 5.82 g 25 mM Tris, 2.93 g 192 mM glycin, pH 9.2, 100 ml Methanol, adjust volume to 1 L dH_2O.
4. Modified Parker medium (MPM): 16.8 ml H 199 10× concentrated, 7.8 ml 7.5% $NaHCO_3$, 100 mg Ca l-Lactate, 40 mg

Na pyruvate, 300 mg HEPES, 20 mg l-Glutamine, 50 U/ml Penicillin K-salt, 50 U/ml Streptomycin, 125 ng/ml Amphotericin B, 15 U/ml P.G. 600 (Intervet, Boxmeer, Holland), 10% oestrus bovine serum, adjust to 200 ml dH_2O.

5. Fertilization medium [10].
6. Menezo B2 medium [11, 12].

2.4 Antibodies

1. Rabbit anti-cullin 1 (Abgent, San Diego, CA).
2. Mouse anti-Skp 1 (Abcam, Cambridge, UK).
3. Rabbit anti-ROC 1 (Abcam).
4. HRP-conjugated donkey anti-rabbit antibody (Jackson Immuno Research, Suffolk, UK).
5. HRP-conjugated donkey anti-mouse antibody (Jackson Immuno Research).
6. Goat anti-rabbit antibody conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, TX).
7. Goat anti-mouse conjugated with Alexa Fluor 594 (Invitrogen Dynal AS, Eugene, OR).

3 Methods

3.1 Oocyte Collection and in Vitro Maturation

1. Wash ovaries briefly in ethanol.
2. Wash ovaries in PBS at 25–30 °C two times.
3. Dissect the follicles (size 5–9 mm) with fine scissors.
4. Puncture the follicles and transfer the cumulus-oocytes complexes (COCs) to 4-well dishes (Nunc, Roskilde, Denmark) with 0.5 ml MPM media without a paraffin overlay and cultivate under a humidified atmosphere at 39 °C with 5% CO_2 for 24 h [10].
5. After 24 h denude part of the MII oocytes by gentle pipetting.
6. Wash MII oocytes in PBS.
7. Use the oocytes for Subheading 3.6 or freeze at −80 °C and use them for Subheading 3.3 or 3.5.
8. Use the rest of COCs for Subheading 3.2.

3.2 In Vitro Fertilization and Embryo Culture

1. After 24 h of maturation wash the rest of COCs in PBS four times.
2. Wash COCs in fertilization medium [10].
3. Transfer up to 40 of COCs to 4-well dishes (Nunc) with 250 μl of fertilization medium.
4. Wash the viable spermatozoa in fertilization medium.

5. Centrifuge spermatozoa at 100 × *g* for 5 min.
6. Count the spermatozoa in a hemocytometer and dilute in the appropriate volume of fertilization medium to concentration of 2×10^6 spermatozoa/ml.
7. Add 250 µl of spermatozoa suspension to fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml.
8. Incubate plates for 20 h at 39 °C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2.
9. At 20 h post fertilization denude presumptive zygotes by gentle pipetting.
10. Transfer groups of 25 zygotes to 25 µl of Menezo B2 medium [11, 12] and cultivate them under the same conditions as in **step 8**.
11. Collect the embryos at 34, 44, 72, 96, 156, and 180 h post fertilization at required stages 2-cell, 4-cell, 8-cell, morula stage, blastocyst stage, and hatched blastocyst stage.
12. Wash embryos in PBS.
13. Use them for Subheading 3.6 or freeze it at −80 °C and use it for Subheading 3.3 or 3.5.

3.3 Poly(A)+ mRNA Extraction

1. Prepare pools of 20 oocytes and embryos at each stage of development.
2. Use Dynabeads mRNA DIRECT Micro Kit (Invitrogen). Unless indicated, the chemicals are included in the kit. Prepare the magnetic rack (PerkinElmer Chemagen Technologie GmbH, Baesweiler, Germany).
3. Bring all buffers except the 10 mM Tris–HCl to room temperature (RT) before use. Store Tris–HCl at 4 °C before use. Warm the incubator to 95 °C.
4. Add 1 pg of luciferase mRNA (Promega, Madison, WI) per oocyte/embryo on ice as an external standard (*see* **Note 1**).
5. Adjust volume to 100 µl/tube by Lysis/Binding buffer and gently mix by vortex.
6. Keep oocyte and embryos in Lysis/Binding buffer for 20 min on ice. Control embryos when they are lysed.
7. *Preparation of Dynabeads:* Shake the bottle with Dynabeads and transfer 20 µl/reaction into new tubes.
8. Place the tube on a magnet for 30 s and discard the supernatant.
9. Remove the tube from magnet, add an equivalent volume of Lysis/Binding Buffer, and resuspend Dynabeads.
10. Place the tube on a magnet for 30 s and discard the supernatant.
11. Remove the tube from magnet, add an equivalent volume of Lysis/Binding Buffer, and resuspend Dynabeads.

12. Transfer 20 µl of Dynabeads/reaction into new tubes.
13. Vortex oocytes/embryos and shortly centrifuge.
14. Transfer the lysed oocytes/embryos to Dynabeads and place tubes on a rotator for 5 min at RT.
15. Place the tubes on the magnet for 30 s and discard the supernatant.
16. Remove the tubes from the magnet and resuspend the samples in 100 µl Washing Buffer A by gentle pipetting.
17. Place the tubes on the magnet for 30 s and discard the supernatant.
18. Remove the tubes from the magnet and resuspend the samples in 100 µl Washing Buffer A by gentle pipetting.
19. Place the tubes on the magnet for 30 s and discard the supernatant.
20. Remove the tubes from the magnet and resuspend the samples in 100 µl Washing Buffer B by gentle pipetting.
21. Transfer the suspension to a new tube.
22. Place the tubes on the magnet for 30 s and discard the supernatant (*see* **Note 2**).
23. Remove the tubes from the magnet and resuspend the samples in 100 µl Washing Buffer B by gentle pipetting.
24. Place the tubes on the magnet for 30 s and discard the supernatant.
25. Remove the tubes from the magnet and resuspend the samples in 3 µl of ice-cold Tris–HCl/embryo.
26. Place the tubes into incubator for 2 min at 95 °C.
27. Place the tubes on the magnet and immediately transfer the supernatant to new tube and place it on ice or store at −80 °C.

3.4 Quantitative RT-PCR

1. Design primers using Beacon Designer 7 (Premier Biosoft, Palo Alto, CA). *See* Table 1.
2. Unless indicated, the chemicals are included in OneStep RT-PCR Kit (Qiagen, Hilden, Germany).
3. Thaw template mRNA, primer solutions dNTP Mix, OneStep RT-PCR buffer and RNase-free water, and place them on ice.
4. Prepare the master mix into one tube composed of: Nuclease-free water (4.55 µl/reaction; Life Technologies, Carlsbad, CA), OneStep Buffer 1× (0.5 µl/reaction), dNTP Mix 400 µM of each (0.5 µl/reaction), forward and reverse primers (both 400 µM; 0.25 µl/reaction), Sybr Green I (1:50,000 of 1000× stock solution, 0.25 µl/reaction; Invitrogen), RNasin Ribonuclease Inhibitor (Promega; 0.2 µl/reaction), OneStep Enzyme Mix (0.5 µl/reaction) (*see* **Note 3**).

Table 1
Information of primers used in this study

Primer	Sequences	Annealing temperature (°C)	Amplicon size (bp)
Cul 1-like (XM_589507.3)	5′-CGG ACT GGA GCC AGA ATC CCA-3′ 5′-GTC TGG GCT TGA GGG GAC ACA-3′	60	178
Cul 1 (NM_001193233.1)	5′-AAC CCC CAC GGA CTC AAG CAG A-3′ 5′-GCC CCT CGA GCT TGG TTT GAC T-3′	60	173
Skp 1 (NM_001034781)	5′-GCC ATC TCC TTG AGC CCT AC-3′ 5′-CAT TTG GCA AGG GGA CTG GA-3′	55	172
Rbx 1 (NM_001034781)	5′-CAG GCG TCC GCT ACT TCT G-3′ 5′-TGT TTT GAG CCA GCG AGA GA-3′	63	93
Luciferase	5′-ACT TCG AAA TGT CCG TTC GG-3′ 5′-ACT TCG AAA TGT CCG TTC GG-3′	55	633

Table 2
RT and PCR conditions used in this study

	Temperature (°C)	Time
Gene	Cul1-like, Cul1/Skp1/Rbx1	Cul1-like, Cul1/Skp1/Rbx1
Reverse transcription	50	30 min
Initial activation	95	15 min
Cycling:		
Denaturation Annealing Extension	94 60/55/63 72	15 s 20 s 30 s
Final extension	72	10 min

5. Mix the master mix thoroughly and distribute appropriate volume (9.0 µl) into the PCR microtubes.
6. Add template mRNA to final volume 12 µl (1 pg–2 µg/reaction). All reactions are prepared in duplicates.
7. Place the tubes into RotorGene 3000 (Corbett Research, Morthlake, Australia). Reaction conditions are as in Table 2.

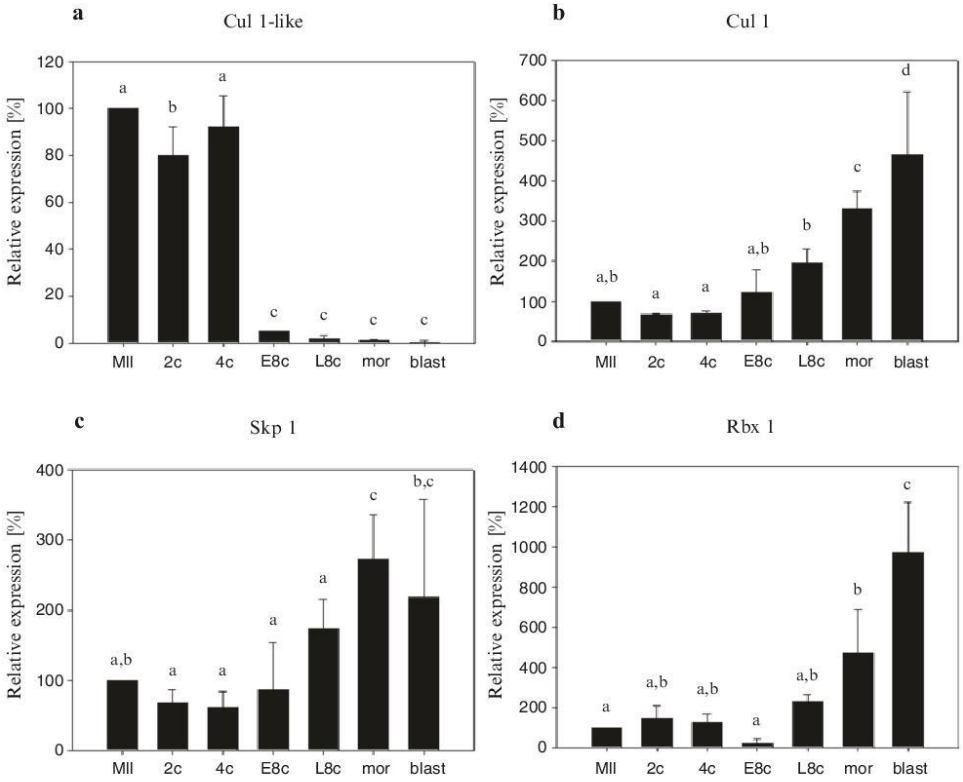


Fig. 1 Relative mRNA expression of invariant members of SCF complex. Untreated embryos. The data were normalized according to the relative concentration of the external standard (luciferase mRNA, 1 pg per oocyte/embryo). (**A**) Cul 1-like, (**B**) Cul1, (**C**) Skp1, (**D**) Rbx1. Bars show ±S.D. [a,b,c,d]Values with different superscripts indicate statistical significance ($P < 0.05$). MII MII stage oocyte, 2c 2-cell stage embryo, 4c 4-cell stage embryo, E8c early 8-cell embryo, L8c late 8-cell stage, mor morula, bl blastocyst

8. Determine the relative concentration of the template in the different samples by comparative quantification in analysis software (Corbett Research), as described in [13]. Normalize the results according to the relative concentration of the external standard (Luciferase) (Fig. 1).

3.5 Western Blot

1. *Day 1*. Prepare gel 4–12% Bis–Tris Gel (*see* **Note 4**).
2. Preparation of samples. Add 2.5 µl LDS Sample Buffer, 1 µl Reducing agent (both Life Technologies), 6 µl deionized water to sample (*see* **Note 5**). Use 25 oocytes/embryos per extract.
3. Boil samples for 3–5 min in distilled water.
4. Transfer to ice.

5. Prepare 1× SDS Running Buffer. Add 35 ml 20× SDS Running Buffer (Life Technologies) to 700 ml deionized water (*see* **Note 6**).
6. Prepare gel to the chamber.
7. Load samples on the gel.
8. Fill chambers with 1× Running Buffer and add 500 µl antioxidant.
9. Run the gel. 200 V constant for about 35 min (*see* **Note 7**).
10. Wash gel in 10% Towbin.
11. Prepare Immobilon P membrane (Millipore Biosciences, Billerica, MA) and filter papers.
12. Prepare the blotting semidry system (Whatman Biometra GmbH, Hoettingen, Germany) and turn on for 28 min at 5 mA/cm^2.
13. After blotting, block the membrane by 3% BSA in TBS-T (CUL 1, RBX 1) or 5% nonfat milk in TBS-T (SKP 1) for 1 h at RT.
14. Incubate with primary antibody. Rabbit anti-cullin 1 1:1000, rabbit anti-ROC 1 1:1000, or mouse anti SKP 1 in 5% nonfat milk, overnight at 4 °C.
15. *Day 2*. Wash membrane in TBS-T 3× in 30 min.
16. Incubate with secondary antibody. HRP-conjugated donkey anti-rabbit or donkey anti-mouse IgG, both 1:7500 in 5% nonfat milk for 1 h at RT.
17. Wash membrane in TBS-T 3× in 30 min.
18. Visualize the proteins with Luminata crescendo Western HRP (Merck Millipore) in dark room.

3.6 Immunofluorescence Analysis

1. *Day 1*. Fix oocyte/embryos in 4% paraformaldehyde for 50 min at 4 °C.
2. Wash in PBS (*see* **Note 8**).
3. Transfer embryos to 0.5% TritonX-100 for 15 min (*see* **Note 9**).
4. Wash embryos in PBS/BSA/sap three times.
5. Block with 2% normal goat serum for 1 h at RT.
6. Wash in PBS/BSA/sap three times.
7. Incubate with primary antibody. Anti-cullin 1 1:100 or anti-SKP1 1:100 or anti-ROC1 1:100, overnight at 4 °C (*see* **Note 10**).
8. *Day 2*. Wash in PBS/BSA/sap 12 times in 1 h.
9. Incubate with secondary antibody goat anti-rabbit with FITC 1:350 or goat anti-mouse with Alexa Fluor 594 1:800 for 1 h at RT in the dark.
10. Wash in PBS/BSA/sap 12 times in 1 h.

11. Wash in PBS.
12. Mount with Vectashield Hardset Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK) (*see* **Note 11**).
13. Check and make a photo of the samples on a fluorescent/confocal microscope (Fig. 2).

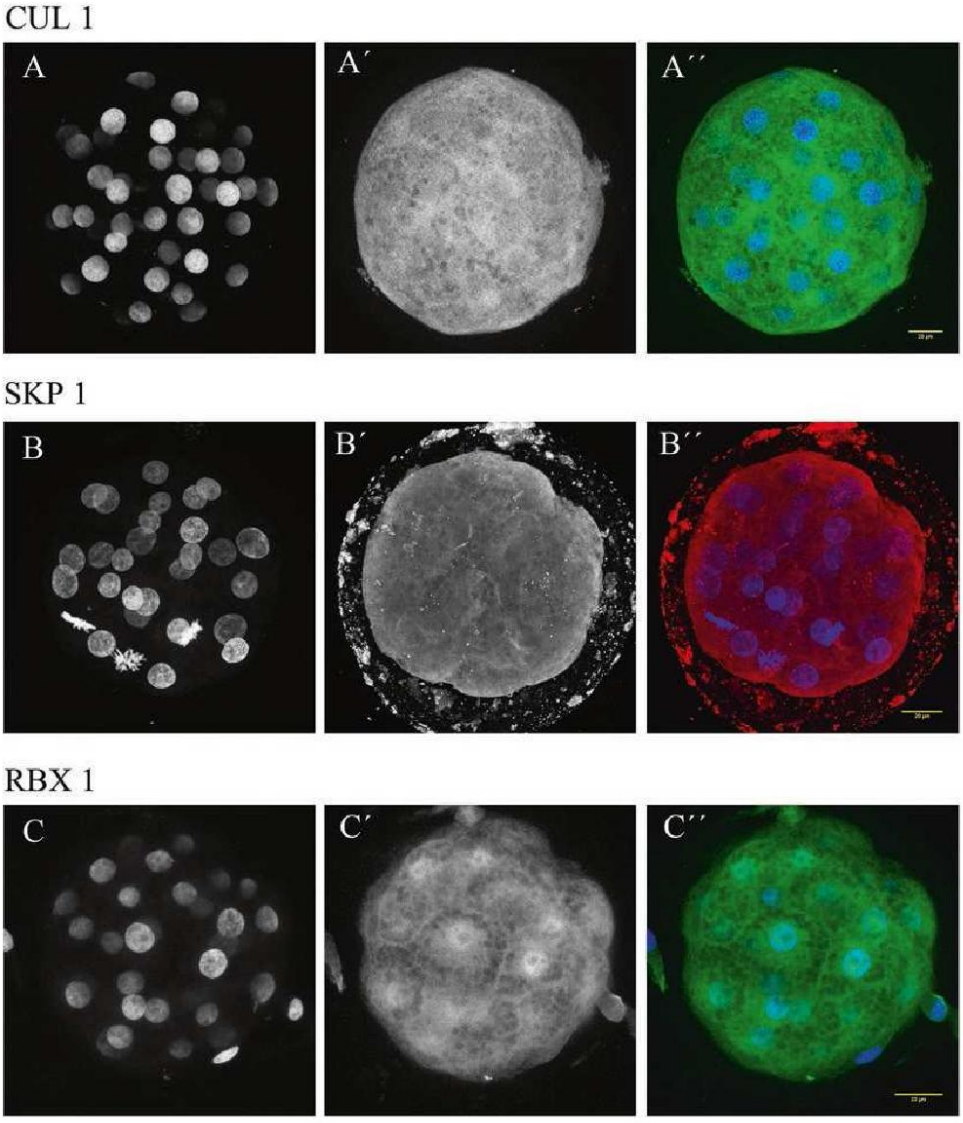


Fig. 2 Confocal laser scanning microscopy of CUL 1, SKP 1, RBX 1 of morula stage embryo. The embryo was labeled with protein-specific antibodies (**A**′) CUL 1, (**B**′) SKP 1, (**C**′) RBX 1, and the nuclei were stained with DAPI (**A**–**C**). In overlaid images CUL 1 (**A**″) and RBX 1 (**C**″) are green, SKP 1 (**B**″) is red, and DAPI (**A**″–**C**″) blue

3.7 In Situ Proximity Ligation Assay (PLA)

The PLA method enables detection and visualization of the protein-protein interaction. If the proteins are in close proximity (<40 nm), it causes cascade of reactions that leads to fluorescent signal. In our case, the interaction of SKP 1 and CUL 1 was observed, which indicates SCF-complex activity [14].

For PLA use Duolink kit (Olink Bioscience, Uppsala, Sweden). Unless otherwise indicated, the chemicals are included in the kit.

1. *Day 1*. Prepare embryos as in Subheading 3.4, **steps 1–4**.
2. Block with 2% normal donkey serum (NDS; Santa Cruz Biotechnology, Santa Cruz, TX), 1 h at RT.
3. Wash in PBS/BSA/sap solution three times.
4. Incubate with primary antibodies—rabbit anti-cullin 1 1:100 together with mouse anti-Skp 1 1:100 overnight at 4 °C (*see* **Note 12**).
5. *Day 2*. Wash embryos in PBS/BSA/sap 12 times in 1 h.
6. *Prepare the PLA probes* by diluting 1:5 in PBS/BSA/sap, and leave it for 20 min at RT (*see* **Note 13**).
7. Add probes and incubate for 1 h at 37 °C.
8. *Ligation*. Wash in PBS/BSA/sap 12 times in 1 h.
9. Prepare the Ligation solution by diluting 1:5 in RNase-free water (*see* **Note 14**).
10. Add ligation solution and incubate for 30 min at 37 °C.
11. *Amplification*. Wash in 1× (Washing Buffer A) WBA three times in 15 min.
12. Prepare polymerization solution by diluting 1:5 in RNase-free water (*see* **Note 15**).
13. *From this step you use light-sensitive reagents. Protect them from light*. Add polymerization solution and incubate for 100 min at 37 °C.
14. Wash six times in 1× WBB in 30 min.
15. Wash in 0.01× WBB (*see* **Note 16**).
16. Wash in PBS.
17. Mount by Vectashield Hardset medium with DAPI (*see* **Note 17**).
18. Check and make a photo of the samples on a fluorescent/confocal microscope (Fig. 3). Dots should represent the interaction of CUL 1 and SKP 1 proteins (*see* **Note 18**).

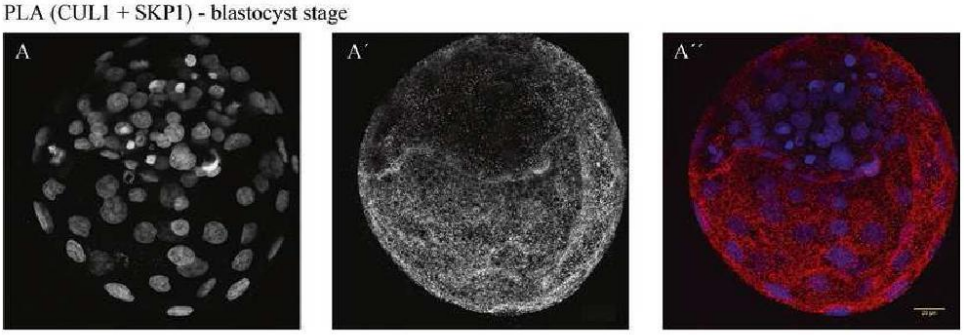


Fig. 3 Confocal laser scanning microscopy of bovine blastocyst after Duolink in situ PLA analysis. PLA signal indicates Cul1-Skp1 interaction (**A′**) and the nuclei were stained with DAPI (**A**). In overlaid (**A″**), PLA signal is red and DNA blue

4 Notes

1. Dilute luciferase in Lysis/Binding buffer.
2. Carefully, in contrast to previous steps, the Dynabeads are not tightly touched.
3. Prepare a volume of master mix 10% greater than required for total number of reactions.
4. We use NuPAGE Bis–Tris Mini Gels (Life Technologies).
5. It is better to prepare the solution together for every sample and then add the appropriate volume to each sample.
6. Use cold water, at 4 °C.
7. 100 V for 10 min at the beginning, then 175–200 V.
8. Fixed embryos can be stored in PBS for up to 3 weeks at 4 °C.
9. Dilute TritonX-100 in PBS.
10. Dilute in PBS/BSA/sap.
11. Before the use, centrifuge the medium for 10 min at 10,000 × *g*.
12. Prepare controls by omitting one or both primary antibodies or using another species-specific secondary conjugate; dilute primary antibodies in PBS/BSA/sap.
13. For a 40 μl reaction take 8 μl of PLA probe MINUS, 8 μl of PLA probe PLUS, and 24 μl of PBS/BSA/sap.
14. For a 40 μl reaction take 8 μl of the 5× Ligation stock, 31 μl of RNAse-free water, and add 1 μl of Ligase immediately before addition to the samples.

15. For a 40 µl reaction take 8 µl of the 5× Amplification stock, 31.5 µl of RNAse-free water, and add 0.5 µl of polymerase immediately before the addition to the samples; light-sensitive reagents.
16. Dilute 1× WBB in RNase-free water; be careful that the embryos tend to stick in WBB.
17. Before the use, centrifuge the medium for 10 min at 10,000 × *g*.
18. Leave it for at least 20 min at 4 °C before checking.

Acknowledgments

We thank R. Sucha and A. Susor for their helpful comments and expert assistance during the experiments and M. Kopcikova, J. Kankova, J. Supolikova, and M. Rakocyova for their technical assistance. This work was supported by the Czech Science Foundation (GACR 13-24730P), Grant Agency of Charles University in Prague (GAUK 1280214) and RVO 67985904.

References

1. Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori KJ, Fujita K, Nakayama H, Fujita J (1993) Non-radioisotopic quantitative RT-PCR to detect changes in mRNA levels during early mouse embryo development. Biochem Biophys Res Commun 195:769–775. doi:10.1006/bbrc.1993.2112
2. DeRenzo C, Seydoux G (2004) A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends Cell Biol 14:420–426. doi:10.1016/j.tcb.2004.07.005
3. Mtango NR, Latham KE (2007) Ubiquitin proteasome pathway gene expression varies in rhesus monkey oocytes and embryos of different developmental potential. Physiol Genomics 31:1–14. doi:10.1152/physiolgenomics.00040.2007
4. Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428. doi:10.1152/physrev.00027.2001
5. Benesova V, Kinterova V, Kanka J, Toralova T (2016) Characterization of SCF-complex during bovine preimplantation development. PLoS One 11:e0147096. doi:10.1371/journal.pone.0147096
6. Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ (1996) SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell 86:263–274
7. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell 91:209–219
8. Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, Wei N, Sun H, Kobayashi R, Zhang H (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. Mol Cell 10:1519–1526
9. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, Cullis CA, Doucette A, Garnsey JJ, Gaulin JL, Gershman RE, Lublinsky AR, McDonald A, Mizutani H, Narayanan U, Olhava EJ, Peluso S, Rezaei M, Sintchak MD, Talreja T, Thomas MP, Traore T, Vyskocil S, Weatherhead GS, Yu J, Zhang J, Dick LR, Claiborne CF, Rolfe M, Bolen JB, Langston SP (2009) An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 458:732–736. doi:10.1038/nature07884
10. Pavlok A, Lucas-Hahn A, Niemann H (1992) Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev 31:63–67. doi:10.1002/mrd.1080310111
11. Menezo Y (1976) Synthetic medium for gamete survival and maturation and for culture of

fertilized eggs. C R Acad Sci Hebd Seances Acad Sci D 282:1967–1970
12. Staessen C, Janssenswillen C, Clerck ED, Steirteghem AV (1998) Controlled comparison of commercial media for human in-vitro fertilization: M n zo B2 medium versus Medi-cult universal and BM1 medium. Hum Reprod 13:2548–2554. doi:10.1093/humrep/13.9.2548
13. Kanka J, Kepkov K, Nemcov L (2009) Gene expression during minor genome activation in preimplantation bovine development. Theriogenology 72:572–583. doi:10.1016/j.theriogenology.2009.04.014
14. Liu J, Furukawa M, Matsumoto T, Xiong Y (2002) NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. Mol Cell 10:1511–1518

4.2. Inhibice Skp1-Cullin-F-box komplexů v průběhu zrání oocytů a preimplantačního vývoje skotu vede k opožděnému vývoji embryí

V následujícím výzkumu jsme se zaměřili na inhibici SCF ligáz u oocytů a embryí skotu. Nejprve jsme inhibovali SCF komplex pomocí RNA interference. Po injikaci specifické dsRNA proti Cul1 bylo množství jeho mRNA signifikantně sníženo. Při kontrole hladiny proteinu CUL1 jsme však zjistili, že protein je i nadále u embryí přítomný a je pravděpodobně příliš stabilní na to, aby mohl být SCF komplex inhibován touto cestou. Z toho důvodu jsme pro další experimenty využili inhibitor MLN4924. MLN4924 (jiným názvem také Pevonedistat) je inhibitor SCF ligáz kontrolovaných neddylací, tedy převážně SCF komplexu obsahující Cul1, v menší míře dalších SCF ligáz obsahující Cul2, Cul3, Cul4A/B nebo Cul5. Byl vyvinut jako potenciální lék proti rakovině (Zhou et al. 2018).

Abychom zjistili, zda je maternální Cul1 (syntetizovaný v průběhu oogezene) potřebný pro oplození a následující embryonální vývoj, byl náš první experiment zaměřen na kultivaci GV oocytů v médiu obsahující inhibitor MLN4924. Rozdíly byly patrné již při expanzi kumulárních buněk. Kumulární buňky ve skupině ošetřené MLN4924 neexpandovaly, během celého zrání se pevně držely na oocytech. Oocyty však byly schopné podstoupit *in vitro* oplození, přestože se signifikantně nižší úspěšností než kontrolní skupina. Popsali jsme také statisticky významný nárůst v množství polyspermních embryí po kultivaci v MLN4924 (51,798 ± 20,721 %) oproti kontrolní skupině (16,52 ± 5,13 %; p=0.016). Při následující kultivaci jsme prokázali zhoršení embryonálního vývoje. Embrya po kultivaci v MLN4924 dosahovala 8buněčného stádia v signifikantně menším počtu a dokonce pouze 8,67 ± 4,59 % embryí se vyvinulo do stádia blastocysty (na rozdíl od kontrolní skupiny, kde dosáhlo stádia blastocysty 20,99 ± 7,69 % embryí).

V dalším experimentu byly inhibovány SCF ligázy u preimplantačních embryí. Embrya skotu byla kultivována v MLN4924 od 4buněčného do pozdního 8buněčného stádia. Tento čas byl zvolen z toho důvodu, že míra degradace maternálních mRNA postupně stoupá a kulminuje v období okolo EGA, což u krav odpovídá právě pozdnímu 8buněčnému stádiu. Pokud by degradace některých maternálních proteinů pomocí SCF ligáz měla podobný průběh, v tomto období by byly SCF ligázy masivně využívány a jejich inhibice by se na následujícím vývoji projevila. Inhibitor byl odmyt v pozdním 8buněčném stádiu a embrya byla kultivována až do stádia blastocysty. Pouze 26,6 ± 2 % embryí ošetřených MLN4924 bylo schopno dosáhnout stádia moruly v porovnání s 57,2 ± 6,98 % kontrolních embryí. 168 hodin po oplození (hpf) bylo schopno dosáhnout stádia blastocysty 22,381 ± 10,852 % kontrolních embryí v porovnání

s pouze 3,889 ± 4,843 % embryí s inhibovanými SCF ligázami. Některá embrya však byla schopná tuto nevýhodu překonat a vyvinout se do blastocyst později, 192 hpf.

U embryí kultivovaných v MLN4924 jsme sledovali aktivaci embryonálního genomu. Zjistili jsme, že aktivace jedněch z nejvýznamnějších markerů EGA (jejich transkripce začíná přesně v tomto období a sledováním jeho exprese je tak možné kontrolovat průběh celé aktivace) PAPOLA a U2AF1 byla opožděná. To naznačuje, že po inhibici SCF ligáz dochází k pozdější aktivaci embryonálního genomu. U těchto embryí jsme následně kontrolovali i množství proteinů pomocí BCA metody. Tato metoda slouží k měření koncentrace proteinů v porovnání s proteinovým standardem na základě kolorimetrické detekce. Prokázali jsme, že u oocytů i embryí po kultivaci v MLN4924 dochází k hromadění proteinů. Abychom odhalili, které proteiny se hromadí, vybrali jsme ty nejdůležitější substráty SCF komplexu a sledovali expresi jejich proteinů pomocí metody western blot. U sledovaných proteinů se nepodařilo prokázat žádné signifikantní navýšení v jejich množství.

Umlčení SCF ligáz v průběhu maturace oocytu i embryonálního vývoje má negativní dopad na expanzi kumulu, oplození, EGA a embryonální vývoj. Dochází k hromadění proteinů, které měly být odstraněny v období před aktivací embryonálního genomu, což mohlo mít, společně s opožděnou EGA negativní vliv i na následující preimplantační vývoj. Prozatím se však nepodařilo prokázat, které proteiny jsou v embryích nahromaděny. K jejich definování povede náš následující výzkum a analýza embryí pomocí hmotnostní spektrometrie.

Publikace:

- **Kinterova V**, Kanka J, Petruskova V, Toralova T. 2019. Inhibition of Skp1-Cullin-F-box complexes during bovine oocyte maturation and preimplantation development leads to delayed development of embryos. Biology of Reproduction **100**:896–906.

Biology of Reproduction, 2019, 100(4), 896–906
doi:10.1093/biolre/ioy254
Research Article
Advance Access Publication Date: 7 December 2018

Research Article

Inhibition of Skp1-Cullin-F-box complexes during bovine oocyte maturation and preimplantation development leads to delayed development of embryos†

Veronika Kinterova[1,2,*], **Jiri Kanka**[1], **Veronika Petruskova**[1,3] **and Tereza Toralova**[1]

[1]Laboratory of Developmental Biology, Institute of Animal Physiology and Genetics Academy of Science of Czech Republic, v.v.i., Libechov, Czech Republic; [2]Department of Veterinary Sciences, Czech University of Life Sciences in Prague, Prague, Czech Republic and [3]Faculty of Science, Charles University in Prague, Prague, Czech Republic

*__Correspondence:__ Institute of Animal Physiology and Genetics, Academy of Science of Czech Republic, v. v. i., Rumburska 89, 277 21 Libechov, Czech Republic. E-mail: kinterova@iapg.cas.cz

†__Grant support:__ This research was supported by grants GACR 13–24730P and CIGA 20172013.
Edited by Dr. Jodi Flaws

Received 26 July 2018; Revised 17 November 2018; Accepted 6 December 2018

Abstract

The mechanism of maternal protein degradation during preimplantation development has not been clarified yet. It is thought that a lot of maternal proteins are degraded by the ubiquitin–proteasome system. In this study, we focused on the role of the SCF (Skp1-Cullin-F-box) complexes during early bovine embryogenesis. We inhibited them using MLN4924, an inhibitor of SCF complex ligases controlled by neddylation. Oocytes matured in MLN4924 could be fertilized, but we found no cumulus cell expansion and a high number of polyspermy after in vitro fertilization. We also found a statistically significant deterioration of development after MLN4924 treatment. After treatment with MLN4924 from the four-cell to late eight-cell stage, we found a statistically significant delay in their development; some of the treated embryos were, however, able to reach the blastocyst stage later. We found reduced levels of mRNA of EGA markers PAPOLA and U2AF1A, which can be related to this developmental delay. The cultivation with MLN4924 caused a significant increase in protein levels in MLN4924-treated oocytes and embryos; no such change was found in cumulus cells. To detect the proteins affected by MLN4924 treatment, we performed a Western blot analysis of selected proteins (SMAD4, ribosomal protein S6, centromeric protein E, P27, NFKB inhibitor alpha, RNA-binding motif protein 19). No statistically significant increase in protein levels was detected in either treated embryos or oocytes. In summary, our study shows that SCF ligases are necessary for the correct maturation of oocytes, cumulus cell expansion, fertilization, and early preimplantation development of cattle.

Summary Sentence

SCF complexes are involved in normal oocyte maturation, polysperm defense, and correct course of preimplantation development.

Key words: ubiquitin–proteasome system, MLN4924, oocyte, cumulus cells, early development, SCF complexes.

Introduction

The early embryonic development of mammals is controlled by maternal mRNA and proteins synthesized during oogenesis. These reserves are used until embryonic genome activation (EGA), a process that occurs at a species-specific developmental stage (the late eight-cell stage in cattle [1], four-cell stage in pigs, and two-cell stage in mice [2]). After fertilization, maternal mRNA and proteins are gradually eliminated [3]. The degradation of maternal mRNA is a gradual process, which peaks around the major wave of EGA [4], but the mechanism underlying the elimination of maternal proteins remains unknown. Two main mechanisms are suggested to be involved in maternal protein degradation—autophagy and the ubiquitin–proteasome system (UPS). Although previous studies suggest the essential participation of autophagy in early embryogenesis [5], UPS seems to play a crucial role in maternal protein degradation during preimplantation development. It has been proved that UPS plays an important role in polar body emission, the formation of pronuclei, meiosis resumption, and cell cycle proliferation [6]. The E3 ubiquitin ligase RNF114 is essential for the activation of the NF-KB pathway during EGA [7], and after silencing the E3 ubiquitin ligase Rnf20, the majority of embryos arrest at the morula stage [8].

UPS-based protein degradation is managed by ubiquitin covalently attached to the targeted proteins by the cooperation of three enzymatic complexes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligases. The E3 enzyme, which binds the substrate, mediates the interaction between ubiquitin and the substrate protein [9]. E3 enzymes are responsible for substrate specificity. One of the most abundant families of E3 enzymes is SCF complexes (Skp1-Cullin-F box, reviewed in [10]). This complex consists of three invariant members: Cullin (Cul), Rbx1, Skp1, and one of the F-box proteins, which determines the substrate specificity [11]. SCF ligases are assumed to mediate up to 20% of proteasome-dependent degradation [12]. The SCF complex is activated by neddylation, post-translational modification conjugating the small ubiquitin-like protein NEDD8 to Cul. Deneddylation is caused by the CAND1 (Cullin-associated and neddylation dissociated 1) protein, which binds to Cul and needs to be dissociated to reactivate the SCF complex [13].

According to our previous results, SCF complex activity is necessary throughout the whole preimplantation development. Furthermore, the early activation of Cul1 and Skp1 mRNA expression indicates its necessity for the preparation of embryos for EGA [14]. Therefore, we decided to explore the role of SCF complexes in preimplantation development in more detail. We show here that inhibiting the activity of SCF ligases leads to an increase in protein level and consequently influences both oocyte maturation (including cumulus expansion) and early embryo development.

Materials and methods

IVF and embryo culture

Unless otherwise indicated, the chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO) and plastic from Nunclon (Nunc, Roskilde, Denmark). Bovine embryos were obtained after the in vitro maturation of oocytes and their subsequent in vitro fertilization (IVF) and in vitro culture. Briefly, abattoir-derived ovaries from cows and heifers were collected and transported in thermos containers in sterile saline at about 33°C. The cattle had been slaughtered (Jatky Rosovice spol, s.r.o.; Slaughterhouse Rosovice) for the public edible meat. Those ovaries were discarded without any utilization; hence, an ethics statement in our paper was not required. The follicles with a diameter between 5 and 9 mm were dissected with fine scissors and then punctured. The cumulus–oocyte complexes (COC) were evaluated and selected according to the morphology of the cumulus and subjected to in vitro maturation in TCM 199 (Earle salt) supplemented with 20 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% estrus cow serum (ECS), serum gonadotropin, and chorionic gonadotropin (P. G. 600, 15 U/ml; Intervet, Boxmeer, Holland) without a paraffin overlay in four-well dishes under a humidified atmosphere for 24 h at 39°C with 5% CO_2.

For IVF, the COC were washed four times in phosphate-buffered saline (PBS) and once in Tyrode albumin lactate pyruvate (TALP) fertilization medium, and transferred in groups of up to 30 to four-well dishes containing 250 μl TALP per well. The TALP medium contained 1.5 mg/ml bovine serum albumin (BSA), 30 μg/ml heparin, 0.25 mM sodium pyruvate, 10 mM lactate, and 20 μM penicillamine. One straw with frozen semen from one bull previously tested in the IVF system was thawed in a 40°C water bath, diluted with 2 ml TALP and centrifuged at 3500 g for 10 min. The spermatozoa were layered under 5 × 1 ml TALP. The supernatant with the motile spermatozoa was isolated after 1 h of swim-up at 39°C [15]. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of TALP to give a concentration of 2×10^6 spermatozoa/ml. A 250 μl aliquot of this suspension was added to each fertilization well to obtain a final concentration of 1×10^6 spermatozoa/ml. Plates were incubated under a humidified atmosphere with 5% CO_2–5% O_2 – 90% N_2 for 20 h at 39°C.

At approximately 20 h post fertilization (hpf), presumed zygotes were denuded by gentle pipetting and transferred in groups up to 25 to 25 μl of Menezo B2 medium (Veterinary Research Institute, Brno, Czech Republic) supplemented with 10% ECS and cultured in a humidified atmosphere 5% CO_2–5% O_2–90% N_2 under liquid paraffin (Origio, Malov, Denmark). Germinal vesicle (GV) oocytes were fixed immediately after isolation or further cultivated. The dishes were then examined at 24 h post isolation and 34, 44, 72, 96, 120, 156, and 180 hpf, and metaphase II (MII) oocytes and two-cell (2c), four-cell (4c), early eight-cell (e8c), late eight-cell (L8c), morula, blastocyst, and hatched blastocyst were collected at each time point, respectively.

Cumulus cells were denuded by gentle pipetting of oocytes, subsequently 2x washed by PBS, centrifuged and frozen (each tube contained cumulus cells from 25 oocytes).

MLN4924 treatment

To determine the optimal dose of MLN4924 for our experiment, several concentrations of MLN4924 were used (detail in the Supplementary material section). To inhibit the SCF complex, MLN4924 was added to the culture medium at a concentration of 1 μM from 4c to L8c stage (44–96 hpf). After MLN4924 treatment, the embryos were washed thoroughly and subsequently cultivated in Menezo B2 medium. Part of L8c stage embryos after MLN4924 treatment was washed with PBS, frozen, and stored at –80°C. Control embryos without treatment were collected at the same time interval as their treated counterparts from the same fertilization/cultivation group.

In the case of inhibition of the SCF complex before IVF, oocytes were cultivated in MPM medium with MLN4924 immediately after COC isolation, cultivated for 24 h, then washed with MPM medium

Table 1. Primer details.

Primer	Sequences	Annealing temperature (°C)	Amplicon size (bp)
Cul1 (NM_001193233.1)	5′-CTGAAGTTCTATACTCAACAATG-3′ 5′-ACAATCTCTCCAAGTCACC-3′ [22]	60	162
U2AF1 (FJ415609.1)	5′-GATGTCGAGATGCAGGAACA-3′ 5′-TCTTCTTCACGGCGAAACTT-3′ [17]	58	155
eiF1A (FJ415608.1)	5′-CTCCCAAGTGACTGAGAAAG-3′ 5′-TCACTCTCCTCCTCGCTCTC-3′ [17]	55	163
PAPOLA (FJ386493.1)	5′-GGATGGTCATGGTTGAGGAG-3′ 5′-GGCGTTGTTTTTCAGTTGGT-3′ [17]	57	162

and frozen or thoroughly washed with fertilization medium and in vitro fertilized.

Quantification of mRNA expression

The embryos were washed using PBS in groups of five and stored dry and deep-frozen at –80°C until used. Poly (A)+ mRNA was extracted from the pool of five embryos using a Dynabeads mRNA DIRECT Micro Kit (Invitrogen Dynal AS, Eugene, OR) according to the manufacturer's instruction.

The expression of mRNA was measured by quantitative RT-PCR, and the reaction was performed using OneStep RT-PCR kit (Qiagen, Hilden, Germany) with real-time detection using SybrGreen fluorescent dye. The reaction composition was Qiagen OneStep RT-PCR buffer (1x), dNTP Mix (400 μM of each), forward and reverse primers (both 400 μM; Table 1), SybrGreen (1: 50 000 of 1000x stock solution; Invitrogen), RNasin Ribonuclease Inhibitor (Promega; 0.2 μl), Qiagen OneStep Enzyme Mix (0.5 μM), and template RNA. Reaction conditions were as follows: RT at 50°C for 30 min, initial activation at 95°C for 15 min, cycling: denaturation at 94°C for 15 s, annealing at 60°C for 20 s (Cul1), at 58°C for 20 s (U2 small nuclear RNA auxiliary factor 1, U2AF1), at 55°C for 20 s (eukaryotic translation initiation factor 1A, eiF1A), at 57°C for 20 s (poly(A) polymerase alpha, PAPOLA), and extension at 72°C for 30 s. The final extension step was held for 10 min at 72°C. The real-time RT-PCRs were run in duplicate, with all samples in the same reaction. The experiments were carried out in a RotorGene 3000 (Corbett Research, Morthlake, Australia). Fluorescence data were acquired at 3°C below the melting temperature to distinguish the possible primer dimers. The qRT-PCR data were determined using serial dilutions of fibroblasts RNA, and the standard curve was created using the take-off points. The take-off points were calculated by Internal RotorGene software (Corbett Research) and defined as the cycle at which the second-derivative curve is at 20% of the maximum rate of fluorescence and indicates the transition to the exponential phase (RotorGene 3000 operation manual; Corbett Research). The starting amount of corresponding RNA in analyzed samples was determined by appointing the take-off points to the curve. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. Experiments were repeated at least three times.

Western blotting

Unless otherwise indicated, chemicals were purchased from Sigma. Embryos and oocytes (20 per extract) were lysed with 6 μl of Millipore H_2O and 2.5 μl of 4x lithium dodecyl sulfate, sample buffer NP 0007 and 1 μl reduction buffer NP 0004 (Novex, Thermo Fisher Scientific, Prague, Czech Republic), boiled for 5 min, and subjected to 4–12% SDS-PAGE. Proteins were transferred from gels to an Immobilon P membrane (Millipore Biosciences, Billerica, MA) using a semidry blotting system (Whatman Biometra GmbH, Hoettingen, Germany) for 28 min at 5 mA/cm^2. The blocking of the membranes was performed in 5% nonfat milk in TBS-Tween (TBS-T, 20 mM Tris, pH 7.4, 137 mM NaCl, and 0.5% Tween 20) for SMAD4 (mothers against decapentaplegic homolog 4), protein 27 (P27), RBM19 (probable RNA-binding protein 19), zygote arrest 1 (ZAR1), alpha-tubulin, in 1% nonfat milk in TBS-T for ribosomal protein S6 (RPS6) and centromeric protein E (CENPE) and in 5% BSA in TBS-T for NFKB inhibitor alpha (IKBA) for 1 h. Membranes were incubated overnight with the following primary antibodies: rabbit anti-CENPE (Cell Signaling Technology 14977, Leiden, Netherlands) 1: 500, rabbit anti- IKBA (Cell Signaling Technology 9242S) 1: 1000, rabbit anti-P27 (Abcam ab32034, Cambridge, UK) 1: 1000, rabbit anti-RBM19 (Abcam ab122515) 1: 250, mouse anti-RPS6 (Santa Cruz Biotechnology SC-74459, Santa Cruz, TX) 1: 1000, rabbit anti-SMAD4 (Proteintech 10231-I-AP, Manchester, UK) 1: 7000, rabbit anti-ZAR1 (Bioss Antibodies bs-13549R, Woburn, MA (Supplementary Table 1)) 1: 300, and rabbit anti-alpha-tubulin (Abcam ab52866) 1: 2000. After washing in TBS-T, the membranes were incubated for 1 h with secondary antibody Peroxidase Anti-Rabbit Donkey (711–035-152, Jackson Immuno Research, Suffolk, UK) or Peroxidase Anti-mouse Donkey (715–035-151, Jackson Immuno Research, Suffolk, UK) both in 1: 7500 dilution in 1% nonfat milk/TBS-T, 5% nonfat milk/TBS-T, or in 5%/TBS-T at room temperature. Proteins were visualized by ECL (Amersham, GE Healthcare life science), and films were scanned using a GS-800 calibrated densitometer (Bio-Rad) and quantified using Image J software (http://rsbweb.nih.gov/ij/).

Total protein amount measurement—Pierce BCA Protein Assay

Embryos were lysed by RIPA buffer (composed of: 150 mM NaCl, 5 mM EDTA pH = 8, 50 mM Tris HCl pH = 7.4, 0.5% NP-40, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS). The protein amount was measured using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Prague, Czech Republic) according to the manufacturer's instruction [16]. Both standard dilution series and samples were prepared in duplicated and incubated for 1.5 h at 37°C. Optical density was measured at 562 nm using Synergy HTX multimode reader (BioTek, Swindon, UK).

Polyspermy detection—immunofluorescence

Embryos were fixed in 4% paraformaldehyde for 50 min at 4°C. Fixed embryos were processed immediately or stored in PBS for up to 3 weeks at 4°C. After washing in PBS, the embryos were

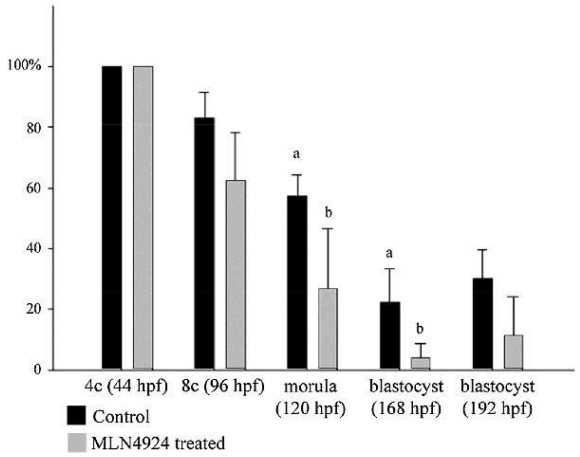


Figure 1. Developmental competence of embryos after the inhibition of a SCF complex using MLN4924 from 4c to 8c embryos. The number of embryos reaching individual developmental stages (y-axis). The number of four-cell-stage embryos is considered as 100%. The developmental competence was followed up during four independent experiments; in total, 150 control and 146 MLN 4924-treated 4c embryos. Bars show mean ± SD (paired t-test). [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$) (4c—four-cell-stage embryos; 8c—eight-cell-stage embryos).

incubated in 0.5% (v/v) TritonX-100 for 15 min. All subsequent steps were done in PBS supplemented with 0.3% (w/v) BSA and 0.05% (w/v) saponin (PBS/BSA/sap). Embryos were blocked with 2% normal goat serum (NGS; Millipore Biosciences; St. Charles, MO) for 1 h and incubated with mouse anti-lamin (Sigma-Aldrich SAB4200236, St. Louis, MO) 1: 150 in PBS/BSA/sap overnight at 4°C (Supplementary Table 1). After thorough washing, the embryos were incubated with goat anti-mouse conjugated with Alexa Fluor 594 1:350 (Invitrogen, Eugene, OR) in PBA/BSA/sap for 1 h at room temperature in the dark. After washing, the nuclei were stained and embryos were mounted in Vectashield HardSet Mounting Medium with DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride, Vector Laboratories, Peterborough, UK). Controls of immunostaining specificity were carried out by omitting the primary antibody or using another species-specific secondary antibody conjugate.

The samples were examined using Leica TCS SP5 (Leica Microsystems AG, Wetzlar, Germany). The images were processed using Image J software.

Statistical analysis

The data were analyzed using SigmaStat 3.0 software (Jandel Scientific, San Rafael, CA). The data were tested for normality, and then paired t-test, Mann–Whitney rank sum test, or t-test was used. $P \leq 0.05$ was considered statistically significant.

Results

Development of embryos after treatment with MLN4924

To investigate the role of SCF complexes during bovine preimplantation development, we used the selective inhibitor of NEDD8-activating enzyme (NAE) MLN4924. NAE is an activator of SCF ligases, and its inhibition prevents the formation of SCF complexes. We did not use the microinjection of cul1 dsRNA, since CUL1 protein is very stable during bovine preimplantation development. After the microinjection of Cul1 dsRNA into zygotes, the Cul1 mRNA was significantly decreased (to 25.31% compared to the controls); nevertheless, the protein level remained unchanged (Supplementary Figures S1 and S2). First, embryos were treated with several concentrations of MLN4924 to determine an optimal concentration for our experiment (Supplementary Figure S3) and 1 μM MLN4924 was selected as the most appropriate concentration. The development of embryos treated with MLN4924 from the 4c to late 8c stage was significantly delayed. Only 26.6 ± 2% (mean ± SD) of MLN4924-treated embryos were able to reach the morula stage compared to 57.2 ± 6.98 ($P = 0.025$) of control embryos. At 168 hpf, 22.381 ± 10.852% (mean ± SD) of control embryos reached the blastocyst stage (the number of four-cell-stage embryos was considered to be 100%). However, only 3.889 ± 4.843% of MLN4924-treated embryos reached the blastocyst stage at this time ($P = 0.01$). On the other hand, some of the embryos treated with MLN4924 were able to reach the blastocyst stage later (Figure 1). Under the microscope, we found no difference in blastocyst quality between them.

To determine whether the maternally inherited CUL1 protein is needed for fertilization and further preimplantation development, we treated the GV oocytes with MLN4924 for 24 h. We have found no expansion of cumulus cells after the MLN4924 treatment of GV oocytes. The condition of cumulus cells remained the same during maturation. Nevertheless, it was possible to fertilize the oocytes. Cumulus cells closely surrounded embryos even after fertilization, in contrast to control embryos gradually losing the surrounding cumulus cells (Figure 2). After IVF, the average ratio of normally fertilized embryos in MLN4924-matured oocytes was significantly reduced to 22.00 ± 16.28% (mean ± SD) compared to 71.57 ± 14.39% of normally fertilized embryos in the control group ($P = 0.014$). In some MLN4924-treated oocytes, a high number

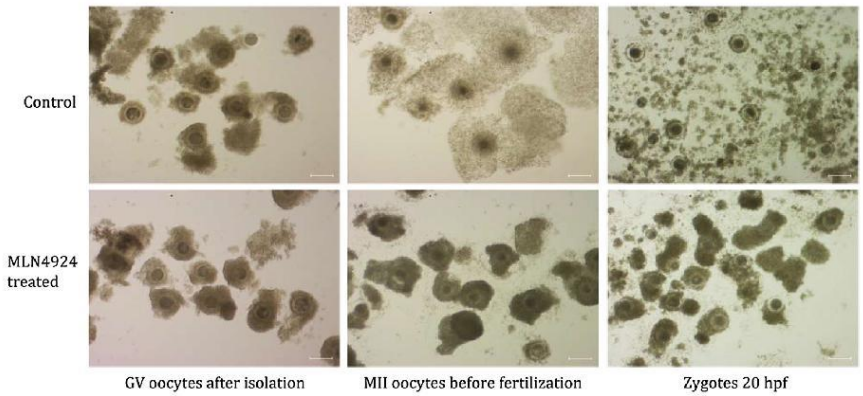


Figure 2. Cumulus cells morphology after MLN4924 treatment. Differences in condition of cumulus cells after MLN4924 cultivation: after isolation, during in vitro maturation of bovine oocytes and 20 h post fertilization.

of sperms were found attached to the zona pellucida, laying in the subzonal space or entering the oocyte. The proportion of unfertilized MLN4924-treated oocytes was significantly higher (26.2% ± 14.70) compared to the control (11.91% ± 14.88) ($P < 0.05$). We observed a significantly higher rate of polysperm embryos in MLN4924-treated oocytes (51.798 ± 20.721%) compared to the control group (16.52 ± 5.13%; $P = 0.016$) (Figure 3A). Lamin staining was used for better visualization of pronuclei (Figure 3B). The development of diploid MLN4924-treated embryos was significantly deteriorated from 8c onwards (8c—$P = 0.005$; morula—$P = 0.015$; blastocyst–$P < 0.05$) and only 8.67% ± 4.59 (mean ± SD) of MLN4924-treated embryos, as compared to 20.99% ± 7.69 (mean ± SD) of control embryos, developed to the blastocyst stage (the number of 2c-stage embryos was to be considered 100%; Figure 3C).

Changes in protein level after MLN4924 treatment

The treatment of embryos using 1 μM MLN4924 from the GV stage to MII stage oocyte and from four-cell-stage to late eight-cell-stage embryos caused a statistically significant increase in protein level ($P < 0.05$ in both cases). It rose to 161.43 ± 122.28 (mean ± SD) in MLN4924-treated oocytes and to 150.17% ± 30.71 (mean ± SD) in MLN4924-treated embryos compared to controls (Figure 4A and B). In contrast, the protein level was not changed in cumulus cells collected after MLN4924 treatment from GV- to MII-stage oocytes ($P > 0.05$) (Figure 4C).

To determine which proteins were affected after the MLN4924 treatment of oocytes and embryos, we performed a Western blot analysis of selected proteins. The proteins were selected as SCF complex substrates or based on their expression level during preimplantation development. SMAD4, RPS6, CENPE, P27, IKBA, ZAR1, and RBM19 were tested. Alpha-tubulin was used as the housekeeper gene. No statistically significant increase in protein level was detected in either treated embryos or oocytes ($P > 0.05$). In embryos two bands after RBM19 staining were detected (130 and 150 kDa). The presence of the higher band is likely caused by phosphorylation or interaction with SUMO2. In MLN4924-treated oocytes, a statistically significant decrease was found in SMAD4 protein level compared to controls ($P < 0.001$; 11.52% ± 3.06; protein level in controls was considered 100%, Figure 5). No statistically significant difference was found in α-tubulin levels ($P > 0.05$).

Delay in EGA initiation after MLN4924 treatment

We monitored the mRNA expression of three EGA markers after the MLN4924 treatment of embryos from 4c to L8c: eiF1A, U2AF1, PAPOLA [17]. The level of expression of U2AF1 and PAPOLA mRNA was significantly lower in MLN4924-treated embryos than in controls (U2AF1: $P = 0.003$; 50.264% ± 21.097; PAPOLA: $P < 0.001$; 47.386% ± 11.806; mean ± SD; the mean of mRNA expression level of controls was considered 100%). The level of eiF1A mRNA expression was not significantly decreased ($P > 0.05$) (Figure 6).

Discussion

The expression profiles of SCF complex members, the existence of two Cul1 variants, the activity of SCF complex during whole preimplantation development, and the currently available data concerning protein degradation during early embryogenesis suggest the potential importance of SCF complexes during early embryonic development [14, 18–22]. To experimentally verify this hypothesis, we used the NAE inhibitor MLN4924. MLN4924 (also known as Pevonedistat) is an inhibitor of SCF ligases controlled by neddylation, primarily Cul1-based SCF ligases or, on a smaller scale, Cul2-, Cul3-, Cul4A/B-, or Cul5- based SCF ligases. It was developed as a potent anti-cancer drug, and to date, most studies with MLN4924 have focused on cancer cells [23]. The first option we considered for the silencing of a gene or connected enzyme in preimplantation embryos was the microinjection of dsRNA into zygotes. This method is well established in early mammalian embryos and frequently used [24–28]. However, the CUL1 protein is highly stable and remains present at a statistically unchanged level even after the silencing of Cul1 mRNA (Supplementary Figures S1 and S2). Thus, MLN4924 treatment was chosen instead.

First, we wanted to know whether oocytes matured with deactivated SCF complexes can be fertilized and establish viable embryos. The oocytes were matured in MLN4924 from the GV to MII stage, and even during this period we noticed a change in

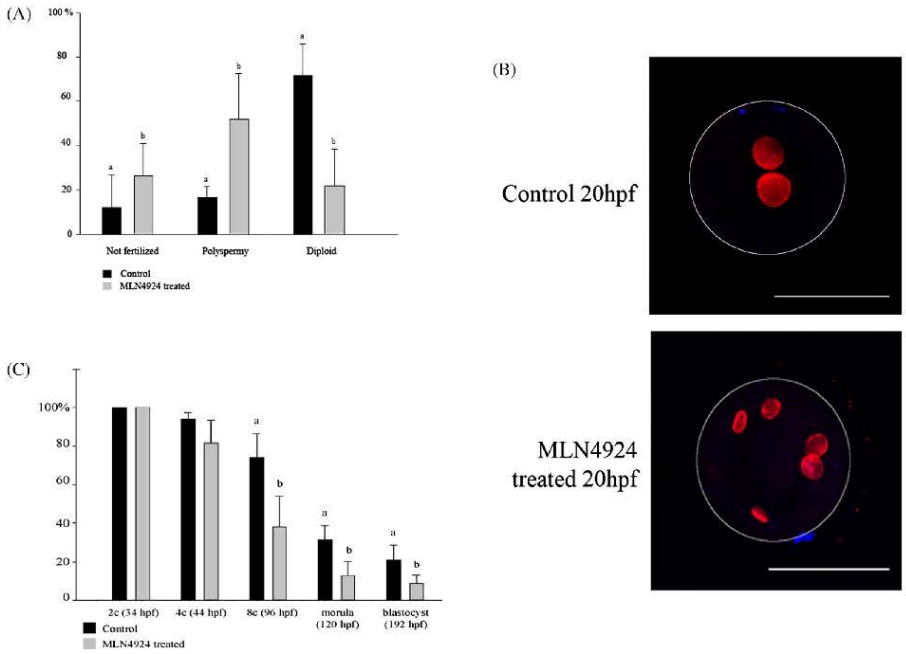


Figure 3. Fertilization and developmental competence of embryos after inhibition of SCF complex using MLN4924 from GV to MII stage. (A) Fertilization ability of oocytes. Relative amounts of embryos belong to following groups: not fertilized, polyspermy, and diploid. In total, 95 control and 105 MLN4924-treated embryos were analyzed. Bars show mean ± SD (paired t-test). [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$). (B) Confocal laser scanning microscopy of diploid control zygote and polyspermy embryo rising from fertilized oocyte treated with MLN4924 during maturation. In total, 95 control embryos and 105 MLN4924-treated embryos were analyzed. Nuclei (DAPI)—blue; lamin—red. Scale bars 100 μm. (C) The developmental competence of embryos arisen from oocytes cultivated in MLN4924 from GV to MII stage. The number of embryos reaching individual developmental stages (y-axis). The number of two-cell-stage embryos is considered as 100%. The developmental competence was followed up during four independent experiments; in total, 207 control embryos and 240 MLN4924-treated 2c embryos. Bars show mean ± SD (paired t-test). [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$). (2c—two-cell-stage embryos; 4c—four-cell-stage embryos; 8c—eight-cell-stage embryos).

cumulus cells morphology. Whilst in controls the cumulus cells expanded and gradually dropped from the zygotes after fertilization, no such change was found in MLN4924-treated oocytes. The role of UPS in the formation of the expanded cumulus is still poorly understood. Yi et al. [29] have shown that after the treatment of GV oocytes with proteasome inhibitor MG132 for 22 h, no oocytes reached the MII stage. In comparison to other proteasome pathway inhibitors such as MG132, which completely block proteasome activity, MLN4924 specifically blocks the activation of SCF complexes. This is probably why almost 74% of MLN4924-treated oocytes could be fertilized, even though only 40.3% zygotes were diploid in comparison to 80% of diploid controls. We assume that the higher polyspermy rate may be due to altered insufficient CG exocytosis. CG exocytosis after fertilization is involved in the ZP-mediated polyspermy blocking and its absence due to inhibition of deubiquinating enzyme (DUB) ubiquitin C-terminal hydrolase L1 (UCHL1) causes increased rates of polyspermy [30]. This suggests that the degradation of proteins by SCF ligases is necessary for the correct course of antipolyspermy defense, in which it likely cooperates with UCHL1 or maybe also other DUBs. Furthermore, we showed that SCF ligases are an essential part of UPS that is involved in cumulus cell expansion. Yi et al. [29] showed that the expansion of cumulus cells is blocked and cumulus cells are firmly attached to oocytes even in the later stages of oocyte maturation in MG132-treated porcine oocytes. This suggests that SCF complexes play an important role in the expansion of cumulus cells. Interestingly, even though the cumulus cells are definitely influenced by the UPS inhibition, we found no change in the total protein level in cumulus cells after MLN4924 treatment compared to controls (Figure 4C).

When we monitored the developmental competence of embryos arising from MLN4924-treated oocytes, we found a statistically significant deterioration of development in comparison to controls. Interestingly, the most statistically significant difference was found at the 8c stage ($P = 0.005$) and the strength of the significance decreased in further stages (morula, $P = 0.016$; blastocyst, $P = 0.048$). Similarly, after the MLN4924 treatment of embryos from 4c to L8c, we found a statistically significant difference between the number of treated embryos and controls at the morula stage ($P = 0.025$) and blastocyst stage at 168 hpf ($P = 0.01$). However, despite there being a clear difference at the blastocyst stage at 192 hpf, it was

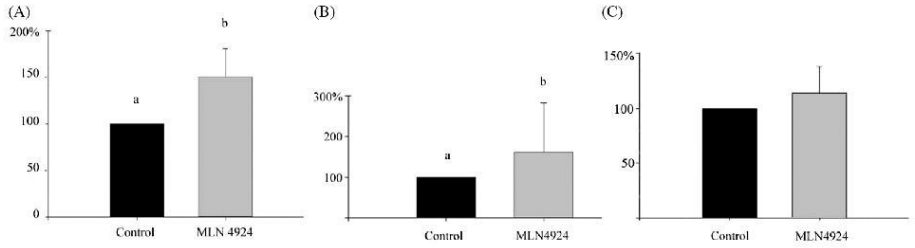


Figure 4. Relative abundance of total protein after treatment with inhibitor MLN4924. (A) Relative protein abundance after treatment from four-cell-stage to late eight-cell-stage embryo. The experiment was repeated four times; in total 30 control embryos and 36 MLN4924-treated embryos. [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$). (B) Relative protein abundance after treatment with inhibitor MLN4924 from GV stage to MII stage oocyte. The experiment was repeated three times; in total 73 control embryos and 82 MLN4924 treated embryos. [a, b]Values with different superscripts indicate statistical significance ($P = 0.032$). (C) Relative protein abundance in cumulus cells after treatment with inhibitor MLN4924 from GV stage to MII stage oocyte. The experiment was repeated three times. No statistically significant difference was found ($P > 0.05$). The total protein amount in control embryos was considered 100%. Bars show mean ± SD (Mann–Whitney rank sum test).

not statistically significant ($P > 0.05$) (Figure 1). This suggests that the degradation of proteins by SCF complexes during oocyte maturation and the early stages of embryogenesis was needed for normal preimplantation development; nevertheless, some embryos after MLN4924 treatment are able to overcome this handicap and develop into blastocysts 24 h later. We suppose that the embryos are temporarily arrested in their development at the 8c stage and resume their growth a few hours after the transfer of embryos to the pure culture medium. Since the deterioration of developmental competence is more distinct after the treatment of GV-MII oocytes than 4c-L8c embryos, normal activity of the SCF complex is apparently more important during oocyte maturation than during the early stages of preimplantation development. The delay in the development of embryos may be related to reduced levels of mRNA of EGA markers PAPOLA and U2AF1A. The level of another EGA marker eiF1A was not significantly changed (Figure 6). The genes involved in protein ubiquitination are activated at 8c in bovines [31], i.e. even before the major wave of EGA, and this also applies to two invariant members of the SCF complex, Cul1 and Rbx1 [14]. Hence, embryonic UPS being responsible for protein degradation is probably necessary for the normal course of EGA. The involvement of proteolysis during EGA was discussed in the work of Stitzel and Seydoux [32] and Liu et al. [33], and the delay in EGA initiation was also found in murine preimplantation embryos treated with MG132 independently of their developmental competence [6]. Our results point to delayed EGA initiation and suggest the importance of protein degradation through the SCF complex. However, since the initiation of eiF1A was not altered in our study in contrast to Shin et al. [6], it seems that for the normal course of EGA the cooperation of multiple parts of the UPS is necessary. The critical stage for embryos arising from MLN4924-treated oocytes is the EGA stage, and the deterioration of development is also obvious in blastocysts. It is very interesting that in embryos that were treated with MLN4924 during the EGA period, the developmental deterioration stops being significant in the late blastocyst stage. This suggests that the preparation for EGA and post-EGA development is already going on during oocyte maturation and the early stages of embryogenesis.

Moreover, we have found a statistically significant increase in total protein level in both treated oocytes and embryos (Figure 4A and B) in contrast to MLN4924-treated cumulus cells (Figure 4C). This shows that as a consequence of SCF complex inhibition, some proteins cannot be degraded and are hoarded. We wanted to know which proteins are affected by the SCF complex inhibition. We tested several SCF complex substrates (SMAD4, RPS6, CENPE, IKBA, P27) [34–38] and other proteins based on their function or expression during preimplantation development (RBM19, ZAR1) [39–41] using Western blot analysis. However, we did not find an increase in protein level in any of these proteins after the deactivation of SCF complexes (Figure 5). An accumulation of P27 was found after the MLN4924 treatment of cell cultures [42, 43], and it is likely that the accumulation of some of the other proteins mentioned above would be found if it was tested in cell cultures in the same way as after SCF complex silencing [34, 44]. After the inhibition of E3 ligase RNF114 in murine preimplantation embryos, the accumulation of only one protein (TGF-beta activated kinase 1, TAB1) was found, even though the authors tested over 9000 proteins [7]. This suggests that the degradation of proteins during oocyte maturation and preimplantation development is subject to strict rules. In somatic cells, mRNAs and proteins can be degraded and synthesized again without limit. In preimplantation embryos, the maternal stores of mRNAs and proteins are stored at least until the EGA stage (late 8c in bovines), and thus those stores need to be conserved. Thus, we suppose that the SCF complexes might be involved in the degradation of the studied proteins even in preimplantation embryos; however, the degradation takes place at a specific time point and/or specific location, similarly to the existence of translational hotspots during oocyte maturation [45]. It is known that the localization of proteins is to some extent driven by their degradation, as proteins are only degraded at a certain location [46]. Interestingly, we found a statistically significant decrease in SMAD4 protein level in MLN4924-treated MII stage oocytes in comparison to controls. We suppose that the decrease of SMAD4 protein amount may be related to atypical maturation of the treated oocytes and cumulus cells as the knockout of SMAD4 gene is connected to cumulus cells defects in mice [47]. In conclusion, we have shown that SCF ligases are necessary for the normal maturation of oocytes, expansion of their cumulus cells, and normal preimplantation development of embryos. Although we found an increase in protein level in both treated oocytes and embryos, we have not found a specific affected protein. To explore which proteins are degraded by SCF complexes during preimplantation development, we plan to subject the treated oocytes and embryos to mass

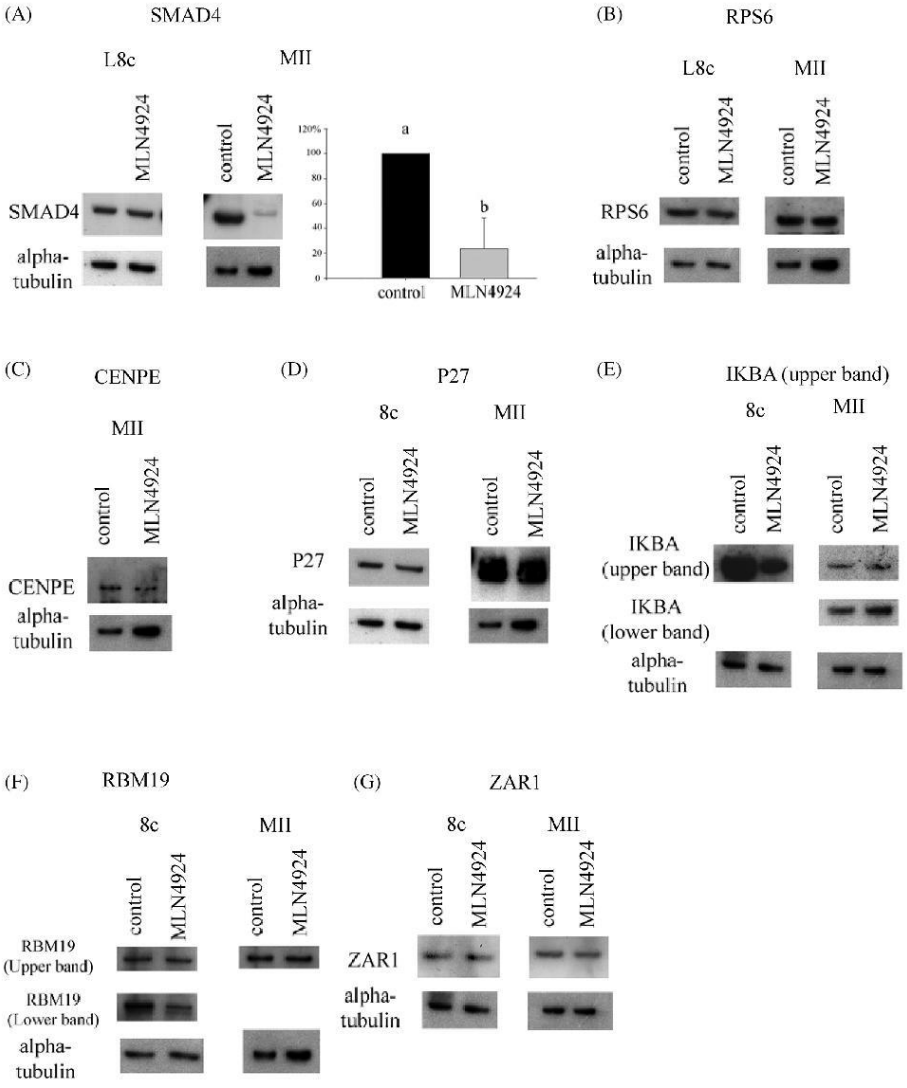


Figure 5. Representative Western blot of level of selected proteins in embryos and oocytes treated with MLN4924. The oocytes were treated during in vitro maturation for 24 h from GV to MII stage; embryos were treated for 48 h from 4c to late eight-cell stage. (A) SMAD4. Bars show mean ± SD. [a,b]Values with different superscripts indicate statistical significance $P < 0.05$. (B) RPS6; (C) CENPE (CENPE in L8c embryos was detected in immeasurable amounts); (D) P27; (E) IKBA; (F) RBM19 (RBM19 was detected at 130 and 150 kDa, in MII the amount of protein at 130 kDa was immeasurable). (G) ZAR1. No statistically significant difference was found in protein level except of SMAD4 in treated MII oocytes (Mann-Whitney rank sum test). All membranes were labeled for α-tubulin as housekeeper gene. Representative Western blot of the studied protein and corresponding α-tubulin staining is shown. The experiments were repeated at least three times (L8c—late eight-cell-stage embryos; MII—MII stage oocytes).

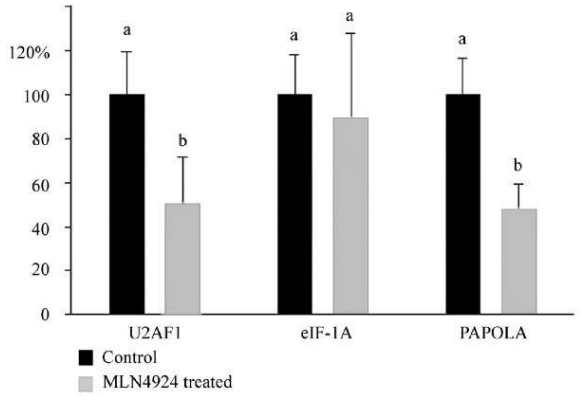


Figure 6. Relative abundance of U2AF1, PAPOLA, and eiF1A mRNA after MLN4924 treatment of 4c to L8c (96 hpf) embryos. The relative abundance (y-axis) represents the amount of corresponding mRNA in a pool of five embryos; mean of mRNA amount in control embryos was considered 100%. In total, 30 control embryos and 25 MLN4924 treated embryos were analyzed for each gene. Bars show mean ± SD (t-test). [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$).

spectrometry analysis. This is a big challenge, since bovine preimplantation embryos are quite a scarce material, and mass spectrometry is very demanding in terms of the amount of material. However, it is probably also the only way to find the SCF complex substrates in oocytes and preimplantation embryos, in which, as our results suggest, protein processing is strictly driven to avoid the degradation of essential maternal stores. This indicates that protein degradation by SCF complexes is an essential process in proper preimplantation development. Incomplete protein degradation and their subsequent higher content leads to developmental delay and to a decrease in the mRNA level of some EGA markers. This developmental delay persists until the early blastocyst stage. Nevertheless, at least some of these embryos are able to overcome this handicap and develop from a morula to a blastocyst about 24 h later. Shin et al. [6], Stitzel and Seydoux [32], and Liu et al. [33] suggest that protein degradation is crucial for EGA, the initiation of which is delayed after UPS inhibition. Our results provide a new piece of the puzzle of the involvement of the UPS in EGA startup, and show the necessity of protein degradation by SCF ligases at this stage, even though other parts of the UPS must also be involved.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplementary table 1. Antibody Table.

Supplementary Figure S1. Relative abundance of cullin 1 mRNA after injection of cullin 1 dsRNA. The relative abundance (y-axis) represents the amount of cullin 1 mRNA in a pool of five embryos at L8c stage, mRNA amount in uninjected embryos was considered 100%. In total, 15 uninjected, 30 GFP dsRNA-injected, and 30 cullin 1 dsRNA-injected embryos were analyzed. Bars show mean ± SD. [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$).

Supplementary Figure S2. Representative Western blot of CUL1 protein level in L8c stage embryos after injection of Cul1 dsRNA. Two different anti-CUL1 antibodies were used (ab199415 and ab75812). Twenty embryos per lane. No statistically significant difference was found in the protein level. The experiments were repeated at least three times (uninjected control, GFP dsRNA injected, Cul1 dsRNA injected embryos).

Supplementary Figure S3. Developmental competence of embryos after cultivation with different concentrations of MLN4924 from 4c to late 8c embryos. (A) 0.3 μM, (B) 1.5 μM, and (C) 3 μM MLN4924 were used. The number of embryos reaching individual developmental stages (y-axis). The number of four-cell-stage embryos is considered as 100%. The developmental competence was followed up during three independent experiments; 360 in total, 118 control, and 242 MLN 4924-treated 4c embryos. Bars show mean ± SD (paired t-test). [a, b]Values with different superscripts indicate statistical significance ($P < 0.05$). The experiments were repeated at least three times (4c—four-cell-stage embryos; 8c—eight-cell-stage embryos).

Acknowledgments

The authors are indebted to A. Susor for helpful comments and the provision of lamin, and CENPE and RPS6 antibodies; to H. Kupcova Skalnikova for expert assistance during the Pierce BCA Protein Assay; to R. Prochazka for helpful comments and to M. Kopcikova, J. Kankova, and J. Supolikova for excellent technical assistance. The authors would also like to thank B.J. Watson-Jones for reading the manuscript and language correction.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References

1. Frei RE, Schultz GA, Church RB. Qualitative and quantitative changes in protein synthesis occur at the 8–16-cell stage of embryogenesis in the cow. *Reproduction* 1989; **86**:637–641.
2. Telford NA, Watson AJ, Schultz GA. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* 1990; **26**:90–100.

3. Minami N, Suzuki T, Tsukamoto S. Zygotic gene activation and maternal factors in mammals. *J Reprod Dev* 2007; **53**:707–715.
4. Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori KJ, Fujita K, Nakayama H, Fujita J. Nonradioisotopic quantitative RT-PCR to detect changes in mRNA levels during early mouse embryo development. *Biochem Biophys Res Commun* 1993; **195**:769–775.
5. Tsukamoto S, Kuma A, Mizushima N. The role of autophagy during the oocyte-to-embryo transition. *Autophagy* 2008; **4**:1076–1078.
6. Shin SW, Tokoro M, Nishikawa S, Lee HH, Hatanaka Y, Nishihara T, Amano T, Anzai M, Kato H, Mitani T, Kishigami S, Saeki K et al. Inhibition of the ubiquitin-proteasome system leads to delay of the onset of ZGA gene expression. *J Reprod Dev* 2010; **56**:655–663.
7. Yang Y, Zhou C, Wang Y, Liu W, Liu C, Wang L, Liu Y, Shang Y, Li M, Zhou S, Wang Y, Zeng W et al. The E3 ubiquitin ligase RNF114 and TAB1 degradation are required for maternal-to-zygotic transition. *EMBO Rep* 2017; **18**:205–216.
8. Ooga M, Suzuki MG, Aoki F. Involvement of histone H2B monoubiquitination in the regulation of mouse preimplantation development. *J Reprod Dev* 2015; **61**: 179–184.
9. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 2002; **82**:373–428.
10. Bosu DR, Kipreos ET. Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Div* 2008; **3**:7.
11. Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* 1996; **86**:263–274.
12. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, Cullis CA, Doucette A et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 2009; **458**:732–736.
13. Liu J, Furukawa M, Matsumoto T, Xiong Y. NEDD8 modification of CUL1 dissociates p120CAND1, an inhibitor of CUL1-SKP1 binding and SCF ligases. *Mol Cell* 2002; **10**:1511–1518.
14. Benesova V, Kinterova V, Kanka J, Toralova T. Characterization of SCF-complex during bovine preimplantation development. *PLoS One* 2016; **11**:e0147096.
15. Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol Reprod Dev* 1992; **31**:63–67.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–254.
17. Verma A, Kumar P, Rajput S, Roy B, De S, Datta TK. Embryonic genome activation events in buffalo (Bubalus bubalis) preimplantation embryos. *Mol Reprod Dev* 2012; **79**:321–328.
18. Benesova V, Kinterova V, Kanka J, Toralova T. Potential involvement of SCF-complex in zygotic genome activation during early bovine embryo development. *Methods Mol Biol* 2017; **1605**:245–257.
19. Dealy MJ, Nguyen KV, Lo J, Gstaiger M, Krek W, Elson D, Arbeit J, Kipreos ET, Johnson RS. Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. *Nat Genet* 1999; **23**:245–248.
20. Wang Y, Penfold S, Tang X, Hattori N, Riley P, Harper JW, Cross JC, Tyers M. Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. *Curr Biol* 1999; **9**:1191–1194.
21. Li B, Ruiz JC, Chun KT. CUL-4A is critical for early embryonic development. *Mol Cell Biol* 2002; **22**:4997–5005.
22. Kepkova KV, Vodicka P, Toralova T, Lopatarova M, Cech S, Dolezel R, Havlicek V, Besenfelder U, Kuzmany A, Sirard MA, Laurincik J, Kanka J. Transcriptomic analysis of in vivo and *in vitro* produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. *Theriogenology* 2011; **75**:1582–1595.
23. Zhou L, Zhang W, Sun Y, Jia L. Protein neddylation and its alterations in human cancers for targeted therapy. *Cell Signal* 2018; **44**:92–102.
24. Toralová T, Benešová V, Kepková KV, Vodička P, Šušor A, Kaňka J. Bovine preimplantation embryos with silenced nucleophosmin mRNA are able to develop until the blastocyst stage. *Reproduction* 2012; **144**:349–359.
25. Toralová T, Susor A, Nemcová L, Kepková K, Kanka J. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. *Reproduction* 2009; **138**:783–791.
26. Nganvongpanit K, Müller H, Rings F, Gilles M, Jennen D, Hölker M, Tholen E, Schellander K, Tesfaye D. Targeted suppression of E-cadherin gene expression in bovine preimplantation embryo by RNA interference technology using double-stranded RNA. *Mol Reprod Dev* 2006; **73**:153–163.
27. Nganvongpanit K, Müller H, Rings F, Hoelker M, Jennen D, Tholen E, Havlicek V, Besenfelder U, Schellander K, Tesfaye D. Selective degradation of maternal and embryonic transcripts in *in vitro* produced bovine oocytes and embryos using sequence specific double-stranded RNA. *Reproduction* 2006; **131**:861–874.
28. Salilew-Wondim D, Hölker M, Rings F, Phatsara C, Mohammadi-Sangcheshmeh A, Tholen E, Schellander K, Tesfaye D. Depletion of BIRC6 leads to retarded bovine early embryonic development and blastocyst formation *in vitro*. *Reprod Fertil Dev* 2010; **22**:564–579.
29. Yi YJ, Nagyova E, Manandhar G, Procházka R, Sutovsky M, Park CS, Sutovsky P. Proteolytic activity of the 26s proteasome is required for the meiotic resumption, germinal vesicle breakdown, and cumulus expansion of porcine cumulus-oocyte complexes matured in vitro. *Biol Reprod* 2008; **78**:115–126.
30. Susor A, Liskova L, Toralova T, Pavlok A, Pivonkova K, Karabinova P, Lopatarova M, Sutovsky P, Kubelka M. Role of ubiquitin C-terminal hydrolase-L1 in antipolyspermy defense of mammalian oocytes. *Biol Reprod* 2010; **82**:1151–1161.
31. Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H, Wolf E. Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments. *Anim Reprod Sci* 2014; **149**:46–58.
32. Stitzel ML, Seydoux G. Regulation of the oocyte-to-zygote transition. *Science* 2007; **316**:407–408.
33. Liu C, Ma Y, Shang Y, Huo R, Li W. Post-translational regulation of the maternal-to-zygotic transition. *Cell Mol Life Sci* 2018; **75**:1707–1722.
34. Wan M, Tang Y, Tytler EM, Lu C, Jin B, Vickers SM, Yang L, Shi X, Cao X. Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. *J Biol Chem* 2004; **279**:14484–14487.
35. Xiao H, Wang H, Silva EA, Thompson J, Guillou A, Yates JR, Jr, Buchon N, Franc NC. The Pallbearer E3 ligase promotes actin remodeling via RAC in efferocytosis by degrading the ribosomal protein S6. *Dev Cell* 2015; **32**:19–30.
36. Liu D, Zhang N, Du J, Cai X, Zhu M, Jin C, Dou Z, Feng C, Yang Y, Liu L, Takeyasu K, Xie W et al. Interaction of Skp1 with CENP-E at the midbody is essential for cytokinesis. *Biochem Biophys Res Commun* 2006; **345**:394–402.
37. Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y. Identification of the receptor component of the IκBα–ubiquitin ligase. *Nature* 1998; **396**:590–594.
38. Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H. p27Kip1 ubiquitination and degradation is regulated by the SCFSkp2 complex through phosphorylated Thr187 in p27. *Curr Biol* 1999; **9**:661–S2.
39. Zhang J, Tomasini AJ, Mayer AN. RBM19 is essential for preimplantation development in the mouse. *BMC Dev Biol* 2008; **8**:115.
40. Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Tore S, Succu S, Berlinguer F, Naitana S, Ledda S. Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and *in vitro*-produced preimplantation embryos. *Reprod Fertil Dev* 2008; **20**:908–915.
41. Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbiès-Tran R. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod* 2004; **71**:1359–1366.

42. Zhang Q, Hou D, Luo Z, Chen P, Lv B, Wu L, Ma Y, Chu Y, Liu H, Liu F, Yu S, Zhang J et al. The novel protective role of P27 in MLN4924-treated gastric cancer cells. *Cell Death Dis* 2015; **6**:e1867–e1867.
43. Tong S, Si Y, Yu H, Zhang L, Xie P, Jiang W. MLN4924 (Pevonedistat), a protein neddylation inhibitor, suppresses proliferation and migration of human clear cell renal cell carcinoma. *Sci Rep* 2017; **7**:5599.
44. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Dev Cell* 2003; **4**:799–812.
45. Susor A, Jansova D, Cerna R, Danylevska A, Anger M, Toralova T, Malik R, Supolikova J, Cook MS, Oh JS, Kubelka M. Temporal and spatial regulation of translation in the mammalian oocyte via the mTOR–eIF4F pathway. *Nat Commun* 2015; **6**:6078.
46. DeRenzo C, Seydoux G. A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. *Trends Cell Biol* 2004; **14**:420–426.
47. Pangas SA, Li X, Robertson EJ, Matzuk MM. Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Mol Endocrinol* 2006; **20**:1406–1422.

4.3. Exprese proteinů SKP1, P27 a IκBα u preimplantačních embryí skotu

Jak již bylo naznačeno dříve, není příliš mnoho informací o degradaci maternálních proteinů v průběhu preimplantačního vývoje. Předpokládá se, že jejich odstranění nebude tak rapidní a obecné jako při degradaci maternální mRNA. Degradace některých proteinů může být specifická způsobem, časem i lokalizací. Je pravděpodobné, že některé proteiny mohou být v embryu uchovávány i do dalších vývojových stádií. Proti degradaci mohou být ochráněny různými způsoby. Mezi ně patří třeba tvorba komplexů, kterou můžeme sledovat například u proteinů *zona pellucida* (Lu et al. 2017), které jsou čistě maternálního původu. Jejich mRNA je degradovaná již v průběhu EGA, přestože tyto proteiny jsou nezbytné až do stádia blastocysty. Proto je nutné je v embryu ochránit před případnou degradací (Bebbere et al. 2016). Dalšími modifikacemi proteinů jsou i různé způsoby dočasného maskování známého například u proteinu CENPE během maturace oocytů (Duesbery et al. 1997).

Při naší analýze exprese různých proteinů v průběhu embryonálního vývoje skotu pomocí metody western blot jsme narazili na zajímavý fenomén. Některé proteiny měly větší molekulární hmotnost, než bylo očekáváno. Jednalo se o proteiny SKP1, P27 a IκBα. Všechny tři proteiny mají přímou souvislost s SCF komplexem. Skp1 je přímo jedním z neměnných členů tohoto komplexu (Bai et al., 1996), P27 a IκBα jsou jeho substráty. Pokusy byly se stejným výsledkem zopakovány u všech vývojových stádií embryí a následně kontrolovány na fibroblastech, kde tyto proteiny vykazovaly standardní velikost. To ukazuje na skutečnost, že tyto změny v molekulární hmotnosti mohou být specifické pro preimplantační embrya. Velikost molekulární hmotnosti neodpovídala jejich násobkům, proto je zřejmé, že vyšší hmotnost není výsledkem polymerizace proteinů.

Prozatím není jasné, jaký má tento jev vysvětlení. Předpokládáme však, že důvodem existence vyšších bandů je právě vytváření komplexů, které mají za úkol je uchránit pro postimplantační vývoj před nechtěnou degradací. Je pravděpodobné, že podobný výsledek bude nalezen i u dalších proteinů analyzovaných u preimplantačních embryí.

Publikace:

- **Kinterova V,** Petruskova V, Kanka J, Toralova T. 2019. Finding of bands of higher molecular weight than expected in three proteins in bovine preimplantation embryos. Zygote **27**(3):187-189.

Zygote

cambridge.org/zyg

Finding of bands of higher molecular weight than expected in three proteins in bovine preimplantation embryos

Veronika Kinterova[1,2], Veronika Petruskova[1,3], Jiri Kanka[1] and Tereza Toralova[1]

[1]Laboratory of Developmental Biology, Institute of Animal Physiology and Genetics, Academy of Science of Czech Republic, v.v.i., Libechov, Czech Republic, [2]Department of Veterinary Sciences, Czech University of Life Sciences in Prague, Prague, Czech Republic and [3]Faculty of Science, Charles University in Prague, Prague, Czech Republic

Short Communication

Cite this article: K. Veronika *et al.* (2019) Finding of bands of higher molecular weight than expected in three proteins in bovine preimplantation embryos. *Zygote* **27**: 187–189. doi: 10.1017/S0967199419000133

Received: 16 August 2018
Revised: 30 November 2018
Accepted: 25 February 2019

Keywords:
IκBα; Molecular weight; p27; Preimplantation embryo; Skp1

Address for correspondence:
Veronika Kinterova. Institute of Animal Physiology and Genetics, Academy of Science of Czech Republic, v. v. i., Rumburska 89, 277 21 Libechov, Czech Republic.
E-mail: kinterova@iapg.cas.cz

Summary

We report here the existence of bands of higher molecular weight after western blot analysis in three proteins – Skp1, p27 and IκBα in bovine preimplantation embryos. This finding is specific to preimplantation embryos (from the 2-cell stage to the blastocyst stage) and not differentiated fibroblast cells in which these bands were of expected molecular weight. We suggest that these bands of higher molecular weight represent a complex of proteins that are characteristic of preimplantation embryos.

Introduction

Mammalian preimplantation development, as well as early embryonic development, in non-mammalian species is a very specific period of life. The cell cycle is much shorter, G1 and G2 phases are almost missing and the embryo lives only from maternal stores. Until the event called embryonic genome activation (EGA) or maternal-to-zygotic transition (MZT) the embryo is transcriptionally silent and only maternally derived mRNAs and proteins are present. As both these types of molecules have usually short lifetimes, their processing has to be altered so that they could be stored from oocyte maturation through fertilization to EGA and in some proteins even further. It has been shown that some maternal proteins are stored after EGA (Svarcova *et al.*, 2007; Ohsugi *et al.*, 2008; Toralova *et al.*, 2012). This prolonged stability may be ensured by some posttranslational modification or complex formation. Such complexes of maternal proteins are formed to persist to preimplantation development and are then involved in driving embryogenesis (reviewed in Lei *et al.*, 2013). This represents primarily proteins like zona pellucida proteins or the SCMC (subcortical maternal complex). However it is possible that maternal proteins form complexes or are modified in order to be preserved for preimplantation development. Such masking or modification was found in oocytes in protein CENPE (Duesbery *et al.*, 1997). Moreover, the SCMC has a molecular weight much larger than is expected by the total mass of all participating proteins (669–2000 kDa vs. expected 325 kDa) (Li *et al.*, 2008).

Material and methods

In vitro *fertilization and embryo culture*

Bovine cumulus–oocytes complexes were obtained from abattoir-derived ovaries. The cattle had been slaughtered (Slaughterhouse Rosovice) for publicly edible meat. Those ovaries were discarded without any utilization. Hence, an ethics statement in our paper was not required. The isolated oocytes were subjected to *in vitro* maturation and subsequent fertilization (Toralova *et al.*, 2012). The embryos were collected after an appropriate time of cultivation (Benesova *et al.*, 2016).

Western blotting

Unless otherwise indicated, chemicals were purchased from Sigma. Unlike the anti-p27 and anti-IκBα antibodies (in which was necessary to use 20 embryos per line), anti-Skp1 antibody gave a really intensive signal and therefore we used just six embryos per line. Embryos were lysed in 15 µl of Blue Loading Buffer (772, Cell Signaling Technology, Danvers, MA, USA) with dithiothreitol, boiled for 5 min and subjected to 12% SDS-PAGE. Proteins were

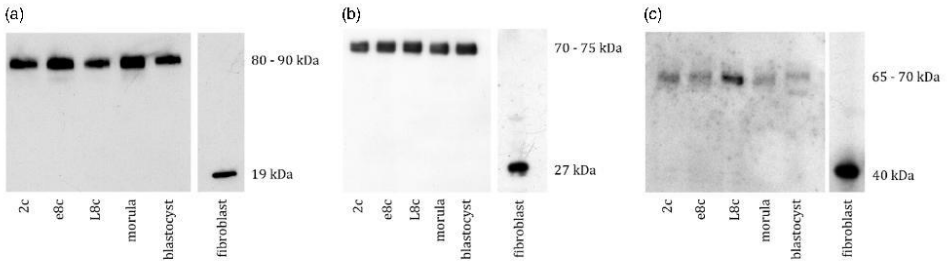


Figure 1. Western blot analysis of bovine preimplantation embryos and bovine fibroblasts. Representative images of western blots of embryos from 2-cell to blastocyst stage: (a) Skp1: 80–90 kDa vs. 19 kDa (six embryos per line). (b) p27: 70–75 kDa vs. 27 kDa (20 embryos per line). (c) IκBα: 65–70 kDa vs. 40 kDa (20 embryos per line). The expected band size responds to the band size in bovine fibroblasts. All experiments were repeated at least three times. 2c, 2-cell stage; e8c, early 8-cell stage, L8c, late 8-cell stage embryos.

transferred from gels to an Immobilon P membrane (Millipore Biosciences, Billerica, MA, USA) using a semidry blotting system (Whatman Biometra GmbH, Hoettingen, Germany) for 28 min at 5 mA/cm^2. Blocking of the membrane was performed in 5% BSA in TBS-Tween buffer (TBS-T, 20 mM Tris, pH 7.4, 137 mM NaCl and 0.5% Tween 20) for IκBα, and in 5% non-fat milk in TBS-T for Skp1 and p27, for 1 h and incubated overnight with following antibodies: IκBα - IκBα Antibody (Cell Signaling Technology 9242, Leiden, The Netherlands) 1:1000 in 5% BSA/TBS-T, p27 - Anti-p27 KIP 1 antibody (Abcam ab32034, Cambridge, UK) 1:1000 in 5% non-fat milk/TBS-T or Skp1–SKP1A monoclonal antibody, clone 1H8 (Abnova M01, Heidelberg, Germany) 1:1000 in 5% non-fat milk/TBS-T. After washing in TBS-T, the membranes were incubated with HRP-conjugated donkey anti-rabbit or donkey anti-mouse IgG antibody (both 1:7500; Jackson Immuno Research, Suffolk, UK) in 5% non-fat milk/TBS-T or in 5 % BSA/TBS-T for 1 h at room temperature. Proteins were visualized with Luminata Crescendo Western HRP (Merck Millipore, Darmstadt, Germany) or ECL (Amersham, GE Healthcare Life Science, UK). Precision Plus Protein™ Dual Color Standards (161-0374, Bio-Rad spol s.r.o., Czech Republic) were used for molecular weight estimation.

Results and discussion

Both embryos and fibroblasts were processed in the same way according to the protocol used in Toralova *et al.* (2012). All experiments were performed at least three times. When performing the western blot analysis, we found the existence of bands of higher molecular weight than expected, in all stages of bovine preimplantation development from the 2-cell stage until the blastocyst stage (representative blot in Fig. 1).

These bands were not possible to be dissolved using dithiothreitol or high temperature (5 min boiling). Simultaneously, analysis of bovine fibroblast cells was performed and the bands emerged at the expected molecular weight. This shows that the bands of higher molecular weight might be specific and typical for preimplantation embryos. However, we cannot exclude that this phenomenon does not exist in another cells, especially non-differentiated, rapidly dividing cells. The described proteins play distinct roles in cell functioning, however all of these have connection to E3-ubiquitin ligase SCF complex (Skp1–Cullin1–F-box protein complex). Skp1 is an invariant member of this complex and is involved in its activation/deactivation control (Bai *et al.*, 1996; Zheng *et al.*, 2002). Besides participation in the ubiquitin-proteasome pathway, it is necessary for correct chromosome segregation and euploidy maintenance in mice (Piva *et al.*, 2002). It is supposed to play an important role during mammalian preimplantation development (Benesova *et al.*, 2016). Incorrect Skp1 expression is involved in development of malignancy (Piva *et al.*, 2002). P27 is a cell cycle regulator especially involved in G1 arrest and in regulation of transcription. Its decreased expression is involved in tumorigenesis and poor prognosis of disease progression (Slingerland & Pagano, 2000). IκBα is involved in NF-κB inhibition by masking its nuclear localization signal and dissociation of NFκB from DNA. Both p27 and IκBα are substrates of the SCF complex, in which Skp1 is incorporated. However, it does not seem that the higher band incidence is related in these three proteins, as the bands are in different heights. It is known that the proteins in early embryos often have multiple isoforms (Tay *et al.*, 2006), however the large difference in molecular weights does not support this explanation. Moreover, the band height is not a multiple of the expected value, so it is not consequence of polymerization.

The reason for the existence of bands of higher molecular weight remains to be elucidated. However, the finding of them in these three proteins speaks for its importance and common occurrence in preimplantation embryos. We suppose that these bands are complex of proteins that arise to preserve them for further stages of preimplantation and may be (as the higher molecular weight bands are present in all preimplantation stages, including blastocysts) to some period of postimplantation development. We assume that similar results can be found also in many other proteins in preimplantation embryos.

Acknowledgements. We would like to thank J. Tyleckova, J. Cervenka, R. Sucha, M. Zizkova and J. Novak for their helpful comments.

Financial support. The work was supported by grant GACR 13-24730P and CIGA 20172013.

Ethical standards. Not applicable.

Statement of interest. None.

References

Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW and Elledge SJ (1996) SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86**, 263–74.

Benesova V, Kinterova V, Kanka J and Toralova T (2016) Characterization of SCF complex during bovine preimplantation development. *PLoS One* **11**, e0147096.

Duesbery NS, Choi T, Brown KD, Wood KW, Resau J, Fukasawa K, Cleveland DW and Vande Woude GF (1997) CENP-E is an essential

kinetochore motor in maturing oocytes and is masked during Mos-dependent, cell cycle arrest at metaphase II. *Proc Natl Acad Sci USA* **94**, 9165–70.

Lei L, Xukun L and Jurrien D (2013) The maternal to zygotic transition in mammals. *Mol Aspects Med* **34**, 919–38.

Li L, Baibakov B and Dean J (2008) A subcortical maternal complex essential for preimplantation mouse embryogenesis. *Dev Cell* **15**, 416–425.

Ohsugi M, Zheng P, Baibakov B, Li L and Dean J (2008) Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. *Development* **135**, 259–69.

Piva R, Liu J, Chiarle R, Podda A, Pagano M and Inghirami G (2002) *In vivo* interference with Skp1 function leads to genetic instability and neoplastic transformation. *Mol Cell Biol* **22**, 8375–87.

Slingerland J and Pagano M (2000) Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* **183**, 10–7.

Svarcova O, Laurincik J, Avery B, Mlyncek M, Niemann H and Maddox-Hyttel P (2007) Nucleolar development and allocation of key nucleolar proteins require *de novo* transcription in bovine embryos. *Mol Reprod Dev* **74**, 1428–35.

Tay TL, Lin Q, Seow TK, Tan KH, Hew CL and Gong Z (2006) Proteomic analysis of protein profiles during early development of the zebrafish, *Danio rerio*. *Proteomics* **6**, 3176–88.

Toralova T, Benesova V, Vodickova Kepkova K, Vodicka P, Susor A and Kanka J (2012) Bovine preimplantation embryos with silenced nucleophosmin mRNA are able to develop until the blastocyst stage. *Reprod Camb Engl* **144**, 349–59.

Zheng JY, Yang XM, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, Wei N, Sun H, Kobayashi R and Zhang H (2002) CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. *Mol Cell* **10**, 1519–26.

4.4. Degradace maternálních proteinů jako opomíjená součást embryonálního vývoje

Ze všech dosavadních poznatků o degradaci maternálních proteinů v průběhu preimplantačního vývoje bylo sepsáno review. Degradace maternálních proteinů je pomalejší, než degradace maternálních mRNA, s větším důrazem na specifičnost, načasování a pravděpodobně i lokalizaci degradace. Zdá se, že zrání oocytů je, v souvislosti s degradací maternálních proteinů, nejdůležitějším obdobím pro normální preimplantační vývoj. Degradace v průběhu preimplantačního vývoje je taktéž důležitá, nicméně bez správného odstraňování proteinů během zrání oocytů nejsou embrya následně schopná normálního vývoje. Pro normální preimplantační vývoj je důležitá jak autofagie, tak ubiquitin – proteasomový systém. Není však jasné, který systém se podílí na degradaci maternálních proteinů.

Shromáždili jsme dostupné informace o odstraňování maternálních proteinů u embryí savců. Tyto informace jsou však značně omezené, proto jsme do review zařadili i vědomosti týkající se nižších živočichů. Naše podklady budou sloužit k následující přípravě matematického modelu, který bude umožňovat přenos poznatků z jednoho modelového organizmu na druhý. Pro správné porozumění mechanizmů zpracování proteinů během časné embryogeneze bude nutné se soustředit zejména na časové období okolo hlavní vlny aktivace embryonálního genomu. Toto období je mnohem dokonaleji popsané u nižších živočichů a na základě těchto poznatků se zdá, že je do velké míry řízeno degradací proteinů. Pravděpodobně nebude docházet k jednotnému, ani rozsáhlému odstraňování proteinů, ale spíše k degradaci jednotlivých skupin nebo specifických proteinů. Toto review nám bude sloužit jako základ k dalšímu výzkumu této problematiky a ulehčí nám práci na připravovaném matematickém modelu, díky kterému budeme schopni určit, jakým způsobem jsou maternální proteiny degradovány u embryí skotu.

Publikace:

- Toralova T, **Kinterova V**, Chmelikova E, Kanka J. 2019. The neglected part of preimplantation development – maternal protein degradation. Cellular and Molecular Life Sciences. (v revizi)

Cellular and Molecular Life Sciences

The neglected part of preimplantation development – maternal protein degradation

--Manuscript Draft--

Manuscript Number:	CMLS-D-19-01006
Full Title:	The neglected part of preimplantation development – maternal protein degradation
Article Type:	Unsolicited review
Corresponding Author:	Veronika Kinterova Ustav zivocisne fyziologie a genetiky Akademie ved Ceske republiky CZECH REPUBLIC
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Ustav zivocisne fyziologie a genetiky Akademie ved Ceske republiky
Corresponding Author's Secondary Institution:	
First Author:	Tereza Toralova
First Author Secondary Information:	
Order of Authors:	Tereza Toralova
	Veronika Kinterova
	Eva Chmelikova
	Jiri Kanka
Order of Authors Secondary Information:	
Funding Information:	Internal Grant Agency of the Czech University of Life Sciences (CIGA 20172013) — Ms. Veronika Kinterova Grantová Agentura České Republiky (GACR 13-24730P) — Ph.D. Tereza Toralova
Abstract:	The degradation of maternal reserves is considered to be one of the most important processes during early embryogenesis. However, the vast majority of papers deals with mRNA degradation and protein degradation is only a very little explored process yet. The aim of this article was to summarize current knowledge about the protein degradation during preimplantation development. In addition to resume of known data concerning mammalian early embryogenesis, we tried to fill the gaps in knowledge by comparison with facts known about protein degradation in early embryos of non-mammalian species. The degradation of maternal proteins is certainly not as universal as maternal RNA degradation. On the contrary, it seems to be driven by very strict rules in terms of specificity and timing. The degradation of maternal proteins is certainly necessary for the normal course of embryonic genome activation (EGA) and several concrete proteins that need to be degraded before major EGA have been already found. Nevertheless, the most important period seems to take place even before preimplantation development – during oocyte maturation. The defects arisen during this period seems to be later irreparable.
Suggested Reviewers:	Poul Hyttel University of Copenhagen, Denmark poh@sund.ku.dk
	Eckhard Wolf Gene Center, LMU, Germany ewolf@lmu.de
	Véronique Duranthon Biologie du Développement et de la Reproduction, INRA veronique.duranthon@jouy.inra.fr

Opposed Reviewers:	

The neglected part of preimplantation development – maternal protein degradation

Tereza Toralova[1], Veronika Kinterova[1,2,*], Eva Chmelikova[2], Jiri Kanka[1]

[1]Laboratory of Developmental Biology, Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Libechov, Czech Republic

[2]Department of Veterinary Sciences, Czech University of Life Sciences in Prague, Prague, Czech Republic

*Correspondence: kinterova@iapg.cas.cz

Summary

The degradation of maternal reserves is considered to be one of the most important processes during early embryogenesis. However, the vast majority of papers deals with mRNA degradation and protein degradation is only a very little explored process yet. The aim of this article was to summarize current knowledge about the protein degradation during preimplantation development. In addition to resume of known data concerning mammalian early embryogenesis, we tried to fill the gaps in knowledge by comparison with facts known about protein degradation in early embryos of non-mammalian species. The degradation of maternal proteins is certainly not as universal as maternal RNA degradation. On the contrary, it seems to be driven by very strict rules in terms of specificity and timing. The degradation of maternal proteins is certainly necessary for the normal course of embryonic genome activation (EGA) and several concrete proteins that need to be degraded before major EGA have been already found. Nevertheless, the most important period seems to take place even before preimplantation development – during oocyte maturation. The defects arisen during this period seems to be later irreparable.

Keywords: ubiquitin – proteasome system, protein degradation, preimplantation development, autophagy, embryonic genome activation

Introduction

The early development of a preimplantation embryo is initially driven by maternally inherited mRNAs and proteins. As development proceeds, the stored stocks are degraded and replaced by embryonally synthesized molecules. There are plenty of articles dealing with maternal RNA processing during early embryonic development (summarized in [1]). The degradation of maternal mRNAs is a gradual process, which peaks around the major wave of embryonic genome activation (EGA) and results in the degradation of the vast majority of maternal mRNAs at this time [2]. On the other hand, it is very challenging to find an article describing the processing of maternally inherited proteins, especially in mammals. Protein degradation is relatively well described during oocyte maturation and fertilization ([3–5] and others) and several proteins such as securin or cyclin B1 are known to be degraded during oocyte maturation [4, 6]. However there have been almost no papers dealing with protein processing during early embryogenesis.

During the pre-EGA stages of preimplantation development the embryos are transcriptionally silent. This indicates that the mRNA must be unusually stable [7], so that it persists even until the EGA stage. Therefore, embryonic

life regulation is driven mostly at the post-transcriptional level with a high impact on the balance of protein synthesis/storage/degradation.

Due to the strict post-transcriptional regulation of stored maternal mRNA, mRNA and protein expression often do not correlate with each other (Stat1, G10 in Xenopus or Nanog in rabbit) [8, 9]. The onset of transcription of a concrete gene often coincides with a decrease in protein level as a result of its maternal mRNA degradation [8]. The levels of other proteins increase during the mid-blastula transition (MBT; an equivalent of EGA in mammals) in Xenopus [8]. Surprisingly, this increase is often caused not only by translation from embryonic but also from maternal mRNA. The maternal mRNA might be translationally silent, and its activation can be postponed as far as the major EGA (e.g. fibronection in Xenopus) [10].

The preimplantation development of mammals is in many ways very specific, and differs from the early embryogenesis of non-mammalian species [11]. The meiotic division of the oocyte is only completed after fertilization, and the cleavage of the early embryo is really slow in comparison to other animals (Figure 1A). In the time it takes mammalian embryos to go from fertilization to implantation, some non-mammalian species have already formed most of their organ primordia. As far as the developmental stage is concerned, the EGA occurs very early in mammalian embryos - the major wave occurs at the 2-cell to 16-cell stage for mammalian species [12–14]. Nevertheless, with respect to the time since fertilization, EGA occurs significantly later in mammals (Figure 1B). At the 8-cell stage (at the same developmental stage for all mammalian species) the mammalian embryo undergoes a process called compaction (Figure 1). During this process, blastomeres that used to loosely attach to each other start to express adhesive molecules such as E cadherin, and the embryo becomes a compact structure. Asymmetry appears after the 8-cell stage with the formation of polar and apolar cells [15, 16], but can still be altered when needed [17, 18]. The cells are still able to adapt to a new environment (reviewed in [19]). The definitive formation of two distinct cell lineages comes at the blastocyst stage with the formation of the inner cell mass (ICM) and trophectoderm (TE). Afterward the blastocyst hatches, which is taken to be the final step of preimplantation development.

In this review, we summarize current knowledge on protein degradation during preimplantation development in mammals. Since a much larger dataset is available on the early embryogenesis of non-mammalian species, we also use this information and try to find a way to transfer this knowledge from non-mammalian to mammalian species.

1. Protein degradation pathways involved in preimplantation development

The degradation pathways of maternal proteins during early embryogenesis are to a large extent unknown. The most attention is paid to the ubiquitin-proteasome system (UPS) and autophagy, nevertheless other minor pathways are involved as well. For example in Xenopus, miRNAs are involved in protein degradation during early embryogenesis [20].

In particular UPS is a highly branched pathway comprise of a lot of different enzymes, many of them involved during preimplantation development. Results to date suggest that maternal protein degradation in embryos is not a mass process, but the degradation of each protein is instead controlled separately. Although the hypotheses and experiments can be largely based on the knowledge gained in somatic cells, there are clearly many specifics in the usage of an enzyme in early embryos for the degradation of a particular protein, its timing or localization.

1.1 Ubiquitin-proteasome system

UPS is a highly specific degradation pathway that primarily deals with the degradation of endogenous proteins. The proteins targeted for degradation are marked using the small protein ubiquitin that is reversibly linked by a

covalent bond to targeted protein [21]. The proteins are polyubiquinated, which directs them to be degraded by the 26S proteasome. The ubiquitination of proteins is performed by three enzyme complexes: E1 – ubiquitin activating enzyme; E2 – ubiquitin-conjugating enzyme and E3 – ubiquitin ligase [21]. While there is only one type of E1 enzyme and a little over 40 types of E2 enzymes, there are more than 500 types of E3 ligases [22]. The plurality of E3 ligases enables the polyubiquitination of different protein targets. The ubiquitinated proteins are rapidly degraded by the proteasome system.

The protein degradation using UPS regulates many cellular functions, including H2B methylation and chromatin remodelling [23, 24], cell cycle progression, the continuous running of transcription [25] and also its termination [26], without which the cell division does not occur. Thus the role of the ubiqutin-proteasome pathway (UPS) in preimplantation development seems to be undeniable, and it is though that a great deal of maternal protein degradation is mediated by the ubiquitin-proteolytic system [27–30]. Moreover, the activity of UPS reflects the embryo quality [28]. Nevertheless, it is still far from clear which E3 ligases are included in the process of maternal protein degradation, as well as which substrates are targeted and when. Moreover, to ensure the flawless course of preimplantation development, ubiquitination without the activation of UPS is also involved in DNA repair, autophagy, transcription and vesicle processing [31].

The amount of proteasomes is mainly regulated at the transcriptional level of its subunits (reviewed in [32]). A decrease in proteasomal activity results in an increased mRNA expression of proteasomal genes and consequently in de novo proteasome formation [33]. However, transcription is inactive during the early stages of preimplantation development, and so the expression of proteasomal genes is not the way proteasome activity is regulated in embryos.

In mice, the polysomal maternal mRNA of genes responsible for ubiquitination is equally present in matured oocytes and one-cell embryos [34]. The embryonic expression of genes involved in protein ubiquitination is activated at the 8-cell stage in bovines; at the same time, the genes required for translation initiation and ribosome biogenesis are activated [35]. The major wave of embryonic genome activation takes place at the late 8c stage in cows. When we focused on the expression of SCF complex members, we found that the mRNA transcription of Cullin1 and Skp1 starts already at the early 8c (i.e. before major EGA) and of Rbx1 at the late 8c stage (EGA stage) [36]. The level of proteins related to UPS sharply increases in mouse zygotes [37], the Cullin1 protein level is the highest at the late 8c and morula stage, the level of Skp1 is the highest at the 4c stage [36]. This shows that the maternal mRNA of genes involved in UPS-based protein degradation is stored and translated until the major wave of EGA, and the expression of embryonic mRNA of these genes is initiated even before the major EGA, so that there is no gap in their protein synthesis. Interestingly, there are large interspecies differences in the expression of UPS genes mRNAs, as was shown by Mtango and Latham [28] after a comparison of mouse and rhesus monkey oocytes and embryos, and also after comparison with our results [36]. It would be very interesting to compare the expression of these genes at the protein level.

Polyubiquinated proteins are accumulated in the embryo around the major EGA - i.e. in mice from the 2-cell stage with rapid degradation at the 4-cell stage [38]. This degradation is consistent with the increase in the chymotrypsin-like activity of the proteasome at the 2-cell stage in mice [38]. However, we found that SCF complex activity does not significantly change during preimplantation development [36]. In preimplantation embryos, proteasomes and ubiquinated proteins are preferentially localized to the nucleus [39–41], active SCF complex and Cullin 1 are mainly localized to the cytoplasm [36, 42]. At the blastocyst stage, ubiquitination is mainly localized to the

trophectoderm. Sutovsky *et al.* [43] found that in bovine and murine blastocysts the diffuse cytoplasmic labelling of ubiquitin was present in both the TE and ICM, however in the TE large granules of ubiquitin were accumulated, whilst these granules were not present in the ICM. Similarly, we found almost no SCF complex activity in the inner cell mass and high SCF complex activity in the TE [36]. On the other hand, the murine deubiquitinase Dub-2 is required for the development of the ICM and hatching of the blastocyst [44], and the localization of proteasomes seems to be the same in ICM and TE (nevertheless, this was observed using ivf-discarded triploid blastocysts; [39]). This indicates that UPS – mediated protein degradation is needed throughout the whole of preimplantation development from oocyte maturation to blastocyst formation. However, the two parts of the UPS – ubiquitination and proteasomal degradation – do not always correlate with each other, and the regulation of protein degradation is driven mainly by the proteasomal activity level rather than the level of ubiquitination in preimplantation embryos.

Moreover, the interaction of a protein with E3-ubiquitin ligase does not necessarily mean its degradation. For example, RecQL4 (RecQ like helicase 4) is ubiquinated by DDB1-Cul4A E3 ubiquitin ligase, which triggers RecQL4 to mediate the repair of double-strand breaks in DNA instead of RecQL4 being degraded [45]. Further, it is associated with the ubiquitin ligases UBR1/UBR2, nevertheless this interaction does not even cause the ubiquitination of RECQL4 [46].

1.1.1. Deubiquitinating enzymes

Deubiquitinating enzymes (DUBs) are thiol proteases that remove the bound ubiquitin from its substrates (proteins, polyubiquitin chains) and thus reverses ubiquitination and enables the renewal of free monoubiquitin ([47, 48] reviewed in [49]). In this way, DUBs not only control (prevent) the substrate degradation, but also recycling of the ubiquitins for further use. It has been shown that silencing or inhibition of a DUB causes affected preimplantation development [50, 51] and others. The deterioration of their preimplantation development is likely mainly caused by the lack of free monoubiquitin (reviewed in [52]). The silencing of DUB USP36 causes decreased mRNA translation in murine morulas. USP36 is a nucleolar protein that is necessary for ribosome biogenesis and RNA processing [51]. To the best of our knowledge, there are no data on USP36 silencing in other mammalian species, however such an effect will likely occur earlier in development, according to the stage at which nucleoli are formed (e.g. at the 8-cell stage in cattle). DUBs certainly play an important role during fertilization in anti-polyspermy defence, specifically the Ubiquitin C-terminal hydrolases (UCHLs) [50]. UCHLs are the most important subgroup of DUBs. Further, there are four other families of DUBs: ubiquitin-specific proteases (USPs), ovarian-tumour domain (OTU DUBs), Machado-Joseph domain (MJD DUBs) and a Jab1/MPN metalloenzyme (JAMM) zinc-dependent metalloprotease (reviewed in [52]), whose role during preimplantation development has however not been elucidated yet.

1.1.1.1. UCHLs

As for oocytes and early embryos, UCHL1 and 3 are the most important members of the UCHL family. UCHL 1 and 3 are necessary for normal oocyte maturation and fertilization and the mechanism of their action seems to be highly conserved (reviewed in [52]). UCHL1 is highly expressed in porcine and bovine oocytes and is localized to the oocyte cortex [50, 53, 54]. This is likely because of its need for cortical granule maturation as part of the later polyspermy block [50]. It is further thought to regulate the level of maternal protein Mater [55]. UCHL3 is localized to the spindle and is probably involved in correct cumulus expansion [52]. Both UCHL1 and 3 are involved in correct polar body extrusion, which ensures leaving enough cytoplasm for the growing oocyte [52,

56]. The incorrect expression of UCHLs during oogenesis and fertilization causes abnormal embryo development, most defects are seen especially during morula compaction and blastocyst formation [56]. A potential role in early embryogenesis may also be played by UCHL2 (mostly referred to as BRCA1-associated protein 1; BAP1), which is needed for the removal of ubiquitin from histone H2B [57]. However its role during preimplantation embryo development has not been studied at all yet.

1.1.2 Ring finger protein 114 (RNF114)

RNF114 is an E3 ubiquitin ligase that is predominantly expressed in oocytes and early embryos, and it is one of the most abundant proteins during the late stage of oocyte maturation [58, 59]. Recently, Yang *et al.* [60] found that RNF114 plays a crucial role in murine preimplantation development through the degradation of TAB1 (TGF-beta activated kinase 1 (MAP3K7) binding protein 1). Embryos with silenced RNF114 arrest at the two-cell stage (i.e. the stage of major EGA), which is likely caused by a defect in NFkB pathway activation during major EGA after TAB1 accumulation [60]. Interestingly, the authors studied more than 9000 proteins, and TAB1 was shown to be the only target of RNF114, whose degradation is necessary for normal preimplantation development [60]. This suggests that the degradation of individual proteins rather than all maternal proteins is important for the initiation of EGA.

1.1.3 Zygote specific proteasome assembly chaperone (ZPAC)

ZPAC is a protein that is specifically expressed in murine gonads, germ cells and early embryos, and not expressed in somatic cells [38, 61]. This is probably because of an increased demand for protein degradation. The expression of ZPAC-like protein in other species has not been found to date [38]. The expression of ZPAC is increased at major EGA and is involved in the degradation of maternal proteins, as it interacts with and stabilizes proteasome assembly chaperone Ump1 (ubiquitin-mediated-proteolysis 1) and promotes 20S proteasome biogenesis [38]. The expression of these proteins is interconnected in the above-mentioned tissues and cells, as the silencing of one of them results in depleting the other [38]. ZPAC replaces PAC proteins that work as proteasome chaperones in somatic cells, probably because of the high abundance of β-precursors of the proteasome in quickly developing cells such as embryos [33, 38, 62]. Its protein level is highest from 12 to 36 hours post fertilization (hpf; peak at 24 hpf) and is almost undetectable after the 8-cell stage in mice. Its mRNA becomes undetectable as soon as 36 hpf [38]. The Ump1 protein remains highly expressed until 48 hpf, but its level sharply decreases thereafter and is also almost undetectable from the 8-cell stage onwards [38]. This is a very early degradation compared to other maternal factors such as Mater, that are present until the blastocyst stage. Both of these proteins are degraded in proteasomes, have a short life-time and are degraded and *de novo* synthesized even during early embryogenesis [38]. The majority of ZPAC knock-down and Ump1-knockdown embryos arrested between the 1- and 2-cell stage, i.e. just before the initiation of major EGA in mice because of the accumulation of β-subunits of the 20S proteasome [38, 61]. Both of these inhibitions cause a decrease in proteasomal activity and an accumulation of polyubiquinated proteins in the treated embryos [38]. ZPAC is also needed for normal spermatogenesis in mice [61, 63].

1.1.4 SCF complex

An SCF complex is an E3 ubiquitin ligase that is formed of Cullin1, Skp1, Rbx1 and an F box protein. The F box determines the specificity of the complex to its substrate. There are also other types of similar ligases based on Cullin 2, 3, 4A, 4B, 5, 7 and 9 that are called Cullin 2 - 9 based CRL (Cullin-RING ubiquitin ligases) complexes, or often Cullin2 - 9 based SCF complexes [64, 65].

SCF complexes are probably involved to a large extent in the protein degradation of maternal proteins, since there are a large number of SCF complexes in mouse oocytes and zygotes [37, 58], it is active throughout preimplantation development, and the embryonic expression of its invariant members is activated no later than the major EGA stage [36]. Moreover, the inhibition of SCF ligase activity causes an increase in total protein level [66]. It is certain that an SCF complex is responsible for the degradation of Cdc6 (cell division cycle 6) before GVBD (germinal vesicle breakdown), CPEB (cytoplasmic polyadenylation element-binding protein) during oocyte maturation, Cdc25A (M-phase inducer phosphatase 1) in Xenopus around the major wave of MBT or Drf1/DFB4B (DBF4 zinc finger B) before the major MBT in Xenopus [67–71]. The processing of Cdc6 and Drf1/DFB4B proteins is discussed in detail in the Specific protein degradation chapter.

1.1.5. Other UPS–related proteins involved in preimplantation development

In addition to the above-mentioned genes, several other proteins related to UPS were found to be necessary for normal preimplantation development. These are mainly E3 ubiquitin ligases (Rnf20, Mdm2 and APC/C) and the ubiquitin conjugation enzyme Ube2a/HR6A. Rnf20 is responsible for H2B monoubiqutination. After the silencing of Rnf20, the ubiquitin ligase of H2B, the majority of embryos arrest at the morula stage and only one third of them develop to the blastocyst stage [72]. This arrest is however not related to protein degradation defects, but to the decrease in H2B monoubiquitination level. Mdm2 causes the degradation of p53, and its knockout causes embryonic lethality due to p53 accumulation [73–75]. Nevertheless, this arrest occurs during the postimplantation period of development around E5.5 (embryonic day; [74, 75]). Anaphase promoting complex (APC/C) prevents securin degradation in mice oocytes and zygotes and causes arrest at the metaphase phase of the cell cycle [76, 77]. APC/C is known to be responsible for cyclin B and securin degradation, which is needed for the cell cycle transition to anaphase [78]. Ube2a/HR6A seems to play the most important role during preimplantation development as the embryos of a Ube2a/HR6A-deficient mother arrest at the 2-cell stage or earlier in mice [79], however the reason for this arrest is unknown. The early arrest of embryos shows that Ube2a/HR6A belongs to the Maternal effect genes group, like the Mater or Filia gene.

1.2. Autophagy

Autophagy is, after UPS, the second most known pathway for degrading proteins, but also other molecules like lipids or small organelles present in the cytoplasm, and it is involved in the degradation of maternal mRNA during early embryogenesis [80–82]. Besides the degradation of unnecessary protein, protein degradation by autophagy is also needed for the recycling of amino acids that are necessary for *de novo* protein synthesis [83]. Autophagy degrades its substrates using lysosomes. It starts with the engulfment of part of the cytoplasm into an autophagosome. The autophagosome then fuses with the lysosome, in which proteins are broken down into amino acids. The regulation of autophagy is driven mainly by mTOR and Atg proteins (reviewed in [84] and [85]. Murine Atg5-/- embryos arrest during the preimplantation phase of development [83], Beclin1 (ortholog of Arg6) -/- embryos arrest after implantation at E7.5 [86] and the inhibition of autophagy causes a significant decrease in blastocyst number in the treated group [87]. Autophagy is also involved in the regulation of pluripotency-associated proteins Oct4, Nanog and Sox2 [87, 88], which together suggest that autophagy plays a crucial role during early embryogenesis. The genes involved in the autophagy pathway are conserved from yeast to humans (reviewed in [89]). The autophagy pathway needs to be started shortly after fertilization, and in mice is active until the late 1-cell stage [83]. Interestingly, this fertilization-induced activation of autophagy is not dependent on

mTORC1 [90] (mTOR is a member of two complexes, mTORC1 and mTORC2 [91]). During the middle 2c stage it becomes reactivated and is active until the 8c stage. After that, the autophagy activity decreases to the basal level and is only reactivated after birth [89]. Nevertheless, autophagy is clearly also important during the 8-cell to implantation period of development [92–94].

A higher rate of autophagy seems to correlate with a higher embryo quality. It was shown that mouse embryos with higher autophagic activity at 4c are of higher quality [95], LC3 (autophagosome marker)-induced autophagy can improve porcine oocyte quality [96], the induction of autophagy in bovine preimplantation embryos increases their developmental competence and on the other hand the inhibition of autophagy leads to a decrease in developmental competence [97]. Autophagy further improves the blastocyst survival rate during implantation [94]. On the other hand, a higher autophagy of oocytes and embryos can result from a poor maturation or developmental condition and is known as pro-death autophagy [93, 98–102]. Autophagy (not only) during preimplantation development is driven by Poly(ADP-ribosyl)ation (PARylation) and PARylation inhibition leads to a decreased autophagic degradation of ubiquinated aggregates, and consequently to a decreased developmental competence of porcine and murine preimplantation embryos, especially at the blastocyst stage [92, 93]. Both the inhibition and induction of autophagy during the earliest stages of preimplantation development (from 1- to 2-cell stage or longer) decreases the number of blastomeres and increases apoptosis in blastocysts in mice. Nevertheless, the induction of autophagy does not influence the number of embryos reaching the blastocyst stage, in contrast to the inhibition of autophagy [87]. This is probably caused by the overexpression of Cdx2 (caudal type homeobox 2), Nanog and Sox2 mRNA in the treated blastocysts. This was true after both the induction and inhibition of autophagy, yet the increase was more significant after the induction of apoptosis, and in addition Pou5f1 (Oct3/4) was also overexpressed [87]. However, the induction/inhibition of autophagy after reaching the 2-cell stage does not affect the development of embryos [87]. The normal operation of autophagy during the division from the 1-cell to the 2-cell stage thus seems to be the most important period of preimplantation development for the production of a normal blastocyst. Surprisingly in pigs, the number of embryos reaching the blastocyst stage was not affected after the inhibition of autophagy from the 1-cell to morula stage, but the number significantly decreased after inhibition from the morula to the blastocyst stage [93]. The authors suggest that only UPS based degradation and not autophagy may be involved in the maternal protein degradation in early stages of porcine preimplantation development [93, 103]. Nevertheless, the development of pig embryos arising from oocytes with an affected autophagy pathway significantly decreases the number and quality of blastocysts [102].

The autophagic activity in embryos declines with maternal age [95] and the level of autophagy in 2-4-cell-stage cloned porcine embryos influences major EGA initiation [82]. Interestingly, the activity of autophagy is dependent on the origin of the embryos. The highest activity of autophagy in porcine cloned embryos was found at the 2-cell stage, however the same was true for 4-cell-stage parthenogenetically activated embryos and 1-cell-stage IVF derived embryos [82, 93]. This is also true for the relocalization of the LC3 autophagosome marker from nuclei to the cytoplasm that coincides with a decrease in autophagic activity [82, 93]. The nuclear localization is likely due to the decondensation of chromatin [93].

Further, autophagy is interconnected with ubiquitination, as it degrades protein complexes that were imperfectly degraded using UPS or in organelle degradation [89]. How ubiquitination is further processed depends on the type of ubiquitin (Ub) chain binding. Chains of 4 or more Ubs on lysine 48 results in processing by UPS, the binding of Ubs on lysine 63 (K63) results in autophagy, the endocytosis of membrane proteins or DNA repair [31, 104,

105]. The timing of K48 and K63 ubiquitination in the peri-fertilization time of C. elegans is different. While UPS-related ubiquitination is present especially before the MII stage, K63 ubiquitination starts from the MII stage onwards and is connected not only to autophagy but also endocytosis [105, 106]. During the degradation of damaged mitochondria, autophagy collaborates with the E3 ubiquitin ligases Parkin and MUL1, which ubiquitinates the mitochondrial membrane protein. In this way, autophagy becomes selective, as the ubiquitination targets the marked mitochondria for lysosomal degradation [107]. The selective degradation is otherwise typical, especially for starvation (reviewed in [52]). Ubiquitination and autophagy also collaborate during paternal mitophagy after fertilization [107–109]. The selective degradation of ubiquitinated proteins is mediated through protein p62, which is a receptor for ubiquitinated proteins for autophagy (reviewed in [110]).

In conclusion, autophagy and ubiquitination clearly intersect with each other in order to correctly degrade/store maternal proteins during preimplantation development. The activity of both these pathways reflects the quality of the embryo [28, 88]. Similarly to UPS-related pathways, autophagy (autophagosomes) is mainly localized to the trophectoderm at the blastocyst stage [36, 43, 94, 111]. However, autophagy during preimplantation development is to a large extent related to the degradation of targets other than proteins (maternal mRNA, lipids, epigenetic changes etc.) [81, 82].

1.3 Endocytosis

Endocytosis is involved in plasma membrane proteins degradation. The selected proteins are first loaded into an intraluminal vesicle and then form multivesicle bodies that fuse with lysosomes [31, 112, 113]. It may be triggered by monoubiquitination or K63 ubiquitination (which also triggers autophagy) [31, 113]. In C. elegans, endocytosis is involved in the selective degradation of plasma membrane proteins after fertilization (e.g. caveolin 1, RME-2, CHS-1, EGG-1) [114–118]. In mammals, the role of endocytosis in maternal plasma membrane protein degradation has not been demonstrated to date, but its role seems to be undeniable, as it is one of the earliest activated pathways in the preimplantation embryo [119].

1.4. Ornithine decarboxylase

Ornithine decarboxylase (ODC1) is a crucially important enzyme for the synthesis of polyamines [120]. Its inhibition causes implantation defects and entry of the murine blastocyst into an embryonic diapause [121]. ODC1 expression is also important during peri-implantation stages in species that do not normally undergo embryonic diapause [122]. The ODC1-/- offspring of ODC1+/- parents are able to implant, but die soon thereafter. This is likely related to the fact that ODC1 expression is needed for ICM expansion [123]. The enzyme is unusual in its specific way of targeting for proteasomal degradation. Instead of being marked with ubiquitin, ODC1 interacts with ornithine decarboxylase antizyme (OAZ), which directs it to be degraded by the 26S proteasome. An intact COOH-terminal part of the protein is needed for the degradation (reviewed in [124]). It seems that ODC1 can be also degraded in an OAZ/COOH-terminal/ubiquitin-independent manner by the 20S proteasome, when not bound to NAD(P)H quinone oxidoreductase (reviewed in [124]). In Xenopus, the maternal protein is stable and is not degraded before MBT [125]. No such data are available for mammalian preimplantation embryos.

2. Waves of protein degradation during early embryogenesis

The first wave of protein degradation occurs even before fertilization, and enables the resumption of meiosis and transition to mitosis. This is when the degradation occurs of proteins like cytoplasmic polyadenylation element-binding protein 1 (CPEB1; involved in translational control) in Xenopus, bovine and mouse oocytes [126–129], Elavl2 in mouse oocytes (translational control) [5], or MEI1 and 2 (microtubule severing complex) in C. elegans

(reviewed in [130]). CPEB is degraded with E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ [67]. MEI1 and 2 are degraded by Cul3-based ubiquitin ligase [131, 132]. The Skp1-Cullin1-Fbox (SCF) complex ligases are suggested to be involved in protein degradation during bovine oocyte maturation and preimplantation development [36, 66, 111], since after the inhibition of SCF-ligases activity during oocyte maturation, an increase in total protein level occurs (see chapter SCF ligases). Interestingly, this inhibition largely influences further preimplantation development even after the transfer of the MII oocytes to standard culture medium for fertilization [66]. The quality of the embryo likely depends on the correct course of protein degradation during oocyte maturation [28, 66]. UPS is also involved in cumulus cell expansion, as the inhibition of UCHL3 or SCF ligases during oocyte maturation prevents cumulus cell expansion [52, 66]. The embryo arising from an oocyte with a defective process of protein storage/degradation may not be able to overcome this handicap, even if its protein processing is appropriate during preimplantation development [28]. Interestingly, the inhibition of UPS using the proteasome inhibitor MG132 in oocytes leads to an increased quality of cloned mammalian embryos [133–138]. The treatment prevents the degradation of cyclin B, maintains MPF activity and cause premature chromosome condensation, which enables a better availability of remodelling and reprogramming of the somatic nucleus and thus better reorganization of chromatin [136, 139]. Furthermore, it likely prevents the inappropriate degradation of proteins essential for initiation of EGA in cloned embryos and regulates the expression of genes involved in histone acetylation [139].

The next wave of protein degradation comes after fertilization of the oocyte. This early degradation depends on both maternally derived UPS and autophagy [41, 83, 90]. After the inhibition of proteasomal activity, polyubiquinated proteins become accumulated after fertilization [140]. UPS is likely involved in the proper reprogramming of the differentiated oocyte into a totipotent zygote after fertilization and its dysfunction is likely the reason for the deteriorated development of somatic cell nuclear transfer embryos [140]. The mouse zygote is enriched in proteins involved in the ubiquitin-proteasome pathway in comparison to MII oocytes, and the amount of proteins in zygotes is decreased compared to MII [37]. In murine MII oocytes, proteins related to ubiquitination and protein degradation amount to 9% [141]. This points to the rapid degradation of maternal proteins after fertilization in mice mentioned in the Ubiquitin proteasome pathway chapter. Moreover, a large set of F-box proteins is present in murine MII and zygotes, which suggests the involvement of an SCF complex in maternal protein degradation in mice [37]. Nevertheless, F-box proteins are involved in many other processes such as epigenetic regulation or reprogramming and its overexpression during the perifertilization period is likely also connected to these UPS-unrelated functions [37]. These results point to the involvement of UPS in post-fertilization protein degradation.

Further degradation is not thoroughly described, and is thought to not be so massive. Instead individual proteins are degraded at specific time points. We suppose that there is a smaller wave of maternal protein degradation before/at the major EGA stage, similarly to such degradation in Xenopus (discussed in the Specific protein degradation chapter). The degradation of maternal proteins is the easiest to explore in genes that are only expressed from maternally derived reserves. For example, the maternal protein Mater (Nlrp5) is stored until the blastocyst stage and hatched blastocyst stage, when it is degraded in mice and bovines, respectively [142, 143]. Gao *et al.* [144] detected a decrease in the protein amount of maternal factors Mater (Nlrp5), Floped or TLE6 even at the 8-cell stage. We suppose that the discrepancy in results is caused by the different method used, as Gao and colleagues used mass spectrometry and Pennetier and Ohsugi and their colleagues used western blot analysis [142–144]. Murine ZPAC and Ump1 are among the earliest degraded maternal proteins. Their degradation occurs at the 8-

cell stage [38] and is discussed in the ZPAC chapter. The degradation of TAB1 occurs even earlier (2-cell stage). This degradation is necessary for the normal course of major EGA [60].

3. Specific protein degradation

Even though there is a large decrease in protein number from MII stage oocytes to zygotes in mouse oocytes [37], not all proteins are degraded before the initiation of EGA. Some of the proteins are stored throughout the whole preimplantation development [143, 145, 146]; reviewed in [147]. In Xenopus, a stable amount of maternally stored proteins is stored long after the MBT, until beating heart [148].

Maternal proteins often form complexes that persist to preimplantation development and are then involved in the regulation of embryogenesis even after major EGA (reviewed in [147]). These are mainly functional complexes of proteins such as Zona pellucida sperm-binding proteins (ZPs) or SCMC (subcortical maternal complex; formed of MATER, FLOPED, TLE6, Filia, PADI6 and likely other proteins [149]. The expression of these genes is exclusively maternal, and mRNAs of its members are degraded at EGA. However, the proteins are stored until the blastocyst stage (reviewed in [150]). Another case of protein modification is the temporary masking of the protein CENPE during oocyte maturation, and it is possible that other proteins are also modified or form complexes to enable their storage to preimplantation development [151]; reviewed in [147]. Some cell cycle regulating proteins (like Stem-loop binding protein or Centromeric protein F) that are expressed in a cell-cycle dependent manner are expressed constantly in oocytes and early embryos [152, 153].

On the other hand, some maternal proteins need to be degraded, so that the embryo can take control over their development. The list of proteins that needs to be degraded before the major wave of EGA/MBT is shown in Table 1. In mammals, the degradation of the TAB1 protein is needed for the normal course of major EGA [60]. Even though it seems that in general maternal protein degradation is not needed for embryonic genome activation, Shin *et al.* [4] have shown that the transient inhibition of the ubiquitin proteasome pathway in murine preimplantation embryos using MG132 delays embryonic genome activation. This delay occurs regardless of whether the embryo is arrested or developing. This suggests that the degradation of maternal protein is highly specific and the degradation of one is just as important as keeping the others.

In Xenopus, five proteins that need to be degraded at major EGA were identified: TopBP1 (a human ortholog of Xenopus Cut5), RecQ4, Treslin, Drf1 (Dumbbell-forming 4; a homolog of mammalian DBF4B) and XCdc6 (Cell division control protein; [8, 154]). All these genes are involved in replication control. Drf1/DBF4B, being part of Cdc7, is essential for the activation of the replication origin complex that the others are part of. Cdc6 is one of the first proteins loaded into the complex. TopBP1/Cut5, RecQ4 (or RecQL4 in mammals) and Treslin are part of the replication pre-initiation complex and are loaded to the replication origin in the second step (reviewed in [155]). Thus, the need for degradation of these proteins is likely connected to the lengthening of the cell cycle after the MBT and less a need for replication initiation. Whether the degradation of these genes is needed for the normal course of major EGA in mammals is to the best of our knowledge unknown. Certainly, these genes are important for mammalian preimplantation development as well. The knockouts are mostly lethal at peri-implantation stages in mammals, however the development of lower animals is enabled far beyond the MBT stage [156 in 157; 158–163]. The only exception is Drf1/DBF4B. Drf1-/- mice are viable, only develop lymphopenia [164] and mutant cell cultures show affected cytoskeletal remodelling [165]. A complete knockout of Drf1/DBF4B was found as a rare sequence variant in humans [166] which further confirms that embryonic development is possible without Drf1/DBF4B expression even though it participates in the manifestation of disease. This similarity of null

phenotypes in these genes suggests that the initiation of embryonic protein synthesis is as important as the degradation of the maternal protein. The differences between mammalian and non-mammalian species are likely due to the difference in developmental speed and must be taken into account when data are transferred between different model organisms. Taken together, it is clear that all the above-mentioned genes are needed for the normal preimplantation development of mammals, however there is no evidence on whether the degradation of maternal protein before major EGA is as necessary as in the Xenopus embryo. Certainly, these genes together form a group of proteins that play a similar role during the earliest phases of development and are processed in a similar manner. The necessity of their degradation is further supported by the overexpression experiments. Embryos overexpressing XCdc6 exhibit a high level of apoptosis [8]. Embryos that combined the overexpression of Cut5, RecQ4, Treslin and Drf1 do not lengthen cell cycles (dependently on protein level) and arrest at stage 17 (i.e. at 17th division) [154]. After the overexpression of Drf1/DBF4B, the viability of the Xenopus embryos decreases after stage 11 [70]. Interestingly, DBF4 overexpression in pre-MBT embryos can suppress the activation of Chk1, but the overexpression is not lethal, in contrast to Drf1 overexpression [70]. Thus, it would be extremely interesting to study the whole group thoroughly and complexly during mammalian preimplantation development.

In Xenopus, two of the above-mentioned proteins – Drf1/DBF4B and Cdc6 – have two distinct variants expressed from two different genes with a switch between those variants around the major MBT. Drf1/DBF4B is replaced by DBF4 before the major wave of MBT and cannot be detected afterwards (Figure 1B, C). Consequently Drf1/DBF4B is present mainly in embryonic cells and DBF4 is found mainly in adult cells [167, 168]. With Cdc6, the Xcdc6A and Xcdc6B variants were found. Only Xcdc6A is functional at early embryonic stages (Xcdc6B is present in early stages, however not functional). It switches around the major MBT stage from XCdcd6A to XCdcd6B and Xcdc6A is undetectable after stage 12 (Figure 1C) and only Xcdc6B is present in somatic cells [169]. Interestingly, Xcdc6A is not expressed in a cell-cycle dependent manner like the somatic Xcdc6B is [169]. Tikhmyanova and Coleman [169], however, suppose that the Cdcd6A/B isoforms may be restricted to Xenopus since they did not find the isoforms in humans, fruit flies, puffer fish and worms in the available genomic databases, and their regulation significantly differs in different organisms [169]. In mammals, a similar switch was found in bovine embryos in Cullin 1, where the maternal variant of cullin 1 mRNA switches to the embryonic (adult) cullin 1 just before the major wave of EGA [36, 42]. The time of protein variant switch is unfortunately unknown. In Xenopus embryos, Drf1/DBF4B is degraded by $SCF^{\beta\text{-}TRCP}$ (Skp1-Cullin1-Fbox complex where β-TRCP represents the F-box protein) at the major MBT. This ensures a Drf1/DBF4B shortage and consequently a lengthening of cell cycles [70]. Thus, we suppose that the switch between DBF4 and Cullin 1 variants might be interconnected and it would be really interesting to find out whether there is a similar switch between Drf1/DBF4B and DBF4 in mammals and study the link between the maternal to embryonic Cullin1 switch and Drf1/DBF4B degradation. The way that Xcdc6 degrade at the major wave of MBT is to the best of our knowledge unknown. However, the degradation of Cdc6 before GVBD is UPS-dependent and is mediated by SCF^{Fbxw7} [71]. (The decrease in Cdc6 protein level in prophase arrest is caused by a decrease in its translation and is UPS independent.)

Similarly to the above-described proteins involved in replication control, Cdc25A is degraded in Xenopus embryos around the major MBT by $SCF^{\beta\text{-}TRCP}$ (Figure 1B, C) [68, 69]. Cdc25A is a mitotic progression inducer and is involved in the resumption of meiosis during mouse oocyte maturation [170]. In Drosophila, a steep decrease in Cdc25A level in cycles 10-13 causes cell cycle lengthening [171]. This is partly caused by Chk1 inhibition, which

in Xenopus mediates the degradation of Drf1 by $SCF^{\beta\text{-}TRCP}$ [70]. In Drosophila, the inhibition of Chk1 is sufficient for the prevention of cell cycle lengthening, which is not true for Xenopus embryos [70, 172].

The necessity of UPS for EGA was also found in mammalian species, as the deactivation of UPS or its part delays the initiation of both the minor and major wave of EGA [4, 38, 60, 61, 66, 140]. However, specific proteins that need to be degraded have not yet been found in mammals. Higuchi and colleagues [140] suppose that the delay is caused by the delayed recruitment of Pol II into the embryo pronuclei. According to their results, the delayed initiation of DNA replication does not seem to cause the delay in major EGA initiation [140]. Together with the results of Collart *et al*. [154], Sun *et al*. [8], Farrell *et al.* [171] and others, this suggests that for the normal course of EGA and further development, the replication rate decrease around major EGA and consequent slowing of development speed is especially important.

4. Concluding remarks

In this review, we summarized current knowledge of the way proteins are processed during preimplantation development. The degradation of maternal proteins is slower than the degradation of maternal RNAs with a large impact on specificity, timing and likely also localization of the degradation. It seems that the most important period for normal preimplantation development in terms of protein degradation occurs already during oocyte maturation. Normal maternal protein degradation during proper preimplantation development is definitely important as well, however without correct protein processing during oocyte maturation, the embryos are not able to develop normally, even under standard conditions. Both autophagy and UPS are necessary for normal preimplantation development. However, the extent to which each of these pathways takes part in maternal protein degradation has not been determined yet.

Since there is limiting data available concerning mammals, the data were also based on a comparison with non-mammalian species and the timing of individual events related to maternal protein degradation in several species was compared. For this purpose, the preparation of a theoretical model that will facilitate the transfer of knowledge from one model organism to the others will be highly beneficial. For a proper understanding of protein processing during preimplantation development, it will be necessary to concentrate especially on the time period around the major wave of EGA. This period is not well described in mammals, and based on the results obtained with lower organisms, it seems to be to a large extent driven by protein degradation. During this period, it is likely that no general or extensive degradation will occur (such as during oocyte maturation or fertilization). We suppose there is a specific degradation of some groups of proteins or even of individual proteins. Identifying such proteins will however be a very challenging and time-consuming work, when we take into account the identification by Yang and colleagues [60] of just one protein whose degradation is necessary for the normal course of major EGA.

Acknowledgements

Supported by grant GACR 13-24730P and Internal Grant Agency of the Czech University of Life Sciences CIGA 20172013.

The authors would like to thank B.J. Watson-Jones for reading the manuscript and language correction.

Conflict of Interest Statement

The authors declare no competing interests.

References:

[1] Svoboda P, Fulka H, Malik R (2017) Clearance of Parental Products. Adv. Exp. Med. Biol. 953:489–535

[2] Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori KJ, Fujita K, Nakayama H, Fujita J (1993) Nonradioisotopic Quantitative Rt-Pcr to Detect Changes in Messenger-Rna Levels During Early Mouse Embryo Development. Biochemical and Biophysical Research Communications 195:769–775.

[3] Karabinova P, Kubelka M, Susor A (2011) Proteasomal degradation of ubiquitinated proteins in oocyte meiosis and fertilization in mammals. Cell and Tissue Research 346:1–9.

[4] Shin SW, Tokoro M, Nishikawa S, Lee HH, Hatanaka Y, Nishihara T, Amano T, Anzai M, Kato H, Mitani T et al. (2010) Inhibition of the ubiquitin-proteasome system leads to delay of the onset of ZGA gene expression. J Reprod Dev 56:655–663.

[5] Chalupnikova K, Solc P, Sulimenko V, Sedlacek R, Svoboda P (2014) An oocyte-specific ELAVL2 isoform is a translational repressor ablated from meiotically competent antral oocytes. Cell Cycle Georget Tex 13:1187–1200.

[6] Huo LJ, Zhong ZS, Liang CG, Wang Q, Yin S, Ai JS, Yu LZ, Chen DY, Schatten H, Sun QY (2006) Degradation of securin in mouse and pig oocytes is dependent on ubiquitin-proteasome pathway and is required for proteolysis of the cohesion subunit, Rec8, at the metaphase-to-anaphase transition. Front Biosci J Virtual Libr 11:2193–2202.

[7] Bachvarova R (1981) Synthesis, turnover, and stability of heterogeneous RNA in growing mouse oocytes. Dev Biol 86:384–392.

[8] Sun L, Bertke MM, Champion MM, Zhu G, Huber PW, Dovichi NJ (2014) Quantitative proteomics of Xenopus laevis embryos: expression kinetics of nearly 4000 proteins during early development. Sci Rep 4:4365.

[9] Henderson GRW, Brahmasani SR, Yelisetti UM, Konijeti S, Katari VC, Sisinthy S (2014) Candidate gene expression patterns in rabbit preimplantation embryos developed in vivo and in vitro. J Assist Reprod Genet 31:899–911.

[10] Lee G, Hynes R, Kirschner M (1984) Temporal and spatial regulation of fibronectin in early Xenopus development. Cell 36:729–740.

[11] Gilbert SF (2000) Developmental Biology. 6th ed. (Sinauer Associates).

[12] Tomanek M, Kopecny V, Kanka J (1989) Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. Anat Embryol (Berl) 180:309–316.

[13] Kanka J (2003) Gene expression and chromatin structure in the pre-implantation embryo. Theriogenology 59:3–19.

[14] Kanka J, Bryova A, Duranthon V, Oudin JF, Peynot N, Renard JP (2003) Identification of differentially expressed mRNAs in bovine preimplantation embryos. Zygote Camb Engl 11:43–52.

[15] Johnson MH, Ziomek CA (1981) The foundation of two distinct cell lineages within the mouse morula. Cell 24:71–80.

[16] Johnson MH, Ziomek CA (1981) Induction of polarity in mouse 8-cell blastomeres: specificity, geometry, and stability. J Cell Biol 91:303–308.

[17] Hiiragi T, Solter D (2004) First cleavage plane of the mouse egg is not predetermined but defined by the topology of the two apposing pronuclei. Nature 430:360–364.

[18] Motosugi N, Bauer T, Polanski Z, Solter D, Hiiragi T (2005) Polarity of the mouse embryo is established at blastocyst and is not prepatterned. Genes Dev 19:1081–1092.

[19] Wennekamp S, Mesecke S, Nédélec F, Hiiragi T (2013) A self-organization framework for symmetry breaking in the mammalian embryo. Nat Rev Mol Cell Biol 14:452–459.

[20] Lund E, Sheets MD, Imboden SB, Dahlberg JE (2011) Limiting Ago protein restricts RNAi and microRNA biogenesis during early development in Xenopus laevis. Genes Dev 25:1121–1131.

[21] Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428.

[22] Fuchs O (2005) The role of ubiquitin-proteasome system in transforming growth factor-ß signaling and its importance in tumorigenesis. Klin Onkol 18.

[23] Muratani M, Tansey WP (2003) How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4:192–201.

[24] Osley MA (2004) H2B ubiquitylation: the end is in sight. Biochim Biophys Acta 1677:74–78.

[25] Lipford JR, Smith GT, Chi Y, Deshaies RJ (2005) A putative stimulatory role for activator turnover in gene expression. Nature 438:113–116.

[26] Gillette TG, Gonzalez F, Delahodde A, Johnston SA, Kodadek T (2004) Physical and functional association of RNA polymerase II and the proteasome. Proc Natl Acad Sci USA 101:5904–5909.

[27] DeRenzo C, Seydoux G (2004) A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends Cell Biol 14:420–426.

[28] Mtango NR, Latham KE (2007) Ubiquitin proteasome pathway gene expression varies in rhesus monkey oocytes and embryos of different developmental potential. Physiol Genomics 31:1–14.

[29] Verlhac MH, Terret ME, Pintard L (2010) Control of the oocyte-to-embryo transition by the ubiquitin-proteolytic system in mouse and C. elegans. Curr Opin Cell Biol 22:758–763.

[30] Suzumori N, Burns KH, Yan W, Matzuk MM (2003) RFPL4 interacts with oocyte proteins of the ubiquitin-proteasome degradation pathway. Proc Natl Acad Sci USA 100:550–555.

[31] Mukhopadhyay D, Riezman H (2007) Proteasome-independent functions of ubiquitin in endocytosis and signaling. Science 315:201–205.

[32] Livneh I, Cohen-Kaplan V, Cohen-Rosenzweig C, Avni N, Ciechanover A (2016) The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. Cell Res 26:869–885.

[33] Meiners S, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel PM, Krüger E (2003) Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of Mammalian proteasomes. J Biol Chem 278:21517–21525.

[34] Potireddy S, Vassena R, Patel BG, Latham KE (2006) Analysis of polysomal mRNA populations of mouse oocytes and zygotes: dynamic changes in maternal mRNA utilization and function. Dev Biol 298:155–166.

[35] Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H, Wolf E (2014) Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments. Anim Reprod Sci 149:46–58.

[36] Benesova V, Kinterova V, Kanka J, Toralova T (2016) Characterization of SCF-Complex during Bovine Preimplantation Development. PloS One 11:e0147096.

[37] Wang S, Kou Z, Jing Z, Zhang Y, Guo X, Dong M, Wilmut I, Gao S (2010) Proteome of mouse oocytes at different developmental stages. Proc Natl Acad Sci USA 107:17639–17644.

[38] Shin SW, Shimizu N, Tokoro M, Nishikawa S, Hatanaka Y, Anzai M, Hamazaki J, Kishigami S, Saeki K, Hosoi Y, et al. (2013) Mouse zygote-specific proteasome assembly chaperone important for maternal-to-zygotic transition. Biol Open 2:170–182.

[39] Wójcik C, Benchaib M, Lornage J, Czyba JC, Guerin JF (2000) Localization of proteasomes in human oocytes and preimplantation embryos. Mol Hum Reprod 6:331–336.

[40] Evsikov AV, de Vries WN, Peaston AE, Radford EE, Fancher KS, Chen FH, Blake JA, Bult CJ, Latham KE, Solter D, Knowles BB (2004) Systems biology of the 2-cell mouse embryo. Cytogenet Genome Res 105:240–250.

[41] Huo LJ, Fan HY, Zhong ZS, Chen DY, Schatten H, Sun QY (2004) Ubiquitin-proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation. Mech Dev 121: 1275–1287.

[42] Kepkova KV, Vodicka P, Toralova T, Lopatarova M, Cech S, Dolezel R, Havlicek V, Besenfelder U, Kuzmany A, Sirard MA, Laurincik J, Kanka J (2011) Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. Theriogenology 75:1582–1595.

[43] Sutovsky P, Motlik J, Neuber E, Pavlok A, Schatten G, Palecek J, Hyttel P, Adebayo OT, Adwan K, Alberio R, et al. (2001) Accumulation of the proteolytic marker peptide ubiquitin in the trophoblast of mammalian blastocysts. Cloning Stem Cells 3:157–161.

[44] Baek KH, Lee H, Yang S, Lim SB, Lee W, Lee JE, Lim JJ, Jun K, Lee DR, Chung Y (2012) Embryonic demise caused by targeted disruption of a cysteine protease Dub-2. PloS One 7:e44223.

[45] Lu H, Shamanna RA, de Freitas JK, Okur M, Khadka P, Kulikowicz T, Holland PP, Tian J, Croteau DL, Davis AJ, et al. (2017) Cell cycle-dependent phosphorylation regulates RECQL4 pathway choice and ubiquitination in DNA double-strand break repair. Nat Commun 8:2039.

[46] Yin J, Kwon YT, Varshavsky A, Wang W (2004) RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. Hum Mol Genet 13:2421–2430.

[47] Larsen CN, Krantz BA, Wilkinson KD (1998) Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry 37:3358–3368.

[48] Amerik AY, Hochstrasser M (2004) Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta 1695:189–207.

[49] Wilkinson KD (2009) DUBs at a glance. J Cell Sci 122:2325–2329.

[50] Susor A, Liskova L, Toralova T, Pavlok A, Pivonkova K, Karabinova P, Lopatarova M, Sutovsky P, Kubelka M (2010) Role of ubiquitin C-terminal hydrolase-L1 in antipolyspermy defense of mammalian oocytes. Biol Reprod 82:1151–1161.

[51] Fraile JM, Campos-Iglesias D, Rodríguez F, Astudillo A, Vilarrasa-Blasi R, Verdaguer-Dot N, Prado MA, Paulo JA, Gygi SP, Martín-Subero JI, et al. (2018) Loss of the deubiquitinase USP36 destabilizes the RNA helicase DHX33 and causes preimplantation lethality in mice. J Biol Chem 293:2183–2194.

[52] Mtango NR, Latham KE, Sutovsky P (2014) Deubiquitinating enzymes in oocyte maturation, fertilization and preimplantation embryo development. Adv Exp Med Biol 759:89–110.

[53] Ellederova Z, Halada P, Man P, Kubelka M, Motlik J, Kovarova H (2004) Protein patterns of pig oocytes during in vitro maturation. Biol Reprod 71:1533–1539.

[54] Massicotte L, Coenen K, Mourot M, Sirard MA (2006) Maternal housekeeping proteins translated during bovine oocyte maturation and early embryo development. Proteomics 6:3811–3820.

[55] Koyanagi S, Hamasaki H, Sekiguchi S, Hara K, Ishii Y, Kyuwa S, Yoshikawa Y (2012) Effects of ubiquitin C-terminal hydrolase L1 deficiency on mouse ova. Reprod Camb Engl 143:271–279.

[56] Mtango NR, Sutovsky M, VandeVoort CA, Latham KE, Sutovsky P (2012) Essential role of ubiquitin C-terminal hydrolases UCHL1 and UCHL3 in mammalian oocyte maturation. J Cell Physiol 227:2022–2029.

[57] Scheuermann JC, Gutiérrez L, Müller J (2012) Histone H2A monoubiquitination and Polycomb repression: the missing pieces of the puzzle. Fly (Austin) 6:162–168.

[58] Zhang P, Ni X, Guo Y, Guo X, Wang Y, Zhou Z, Huo R, Sha J (2009) Proteomic-based identification of maternal proteins in mature mouse oocytes. BMC Genomics 10:348.

[59] Liu C, Ma Y, Shang Y, Huo R, Li W (2018) Post-translational regulation of the maternal-to-zygotic transition. Cell Mol Life Sci CMLS 75:1707–1722.

[60] Yang Y, Zhou C, Wang Y, Liu W, Liu C, Wang L, Liu Y, Shang Y, Li M, Zhou S, et al. (2017) The E3 ubiquitin ligase RNF114 and TAB1 degradation are required for maternal-to-zygotic transition. EMBO Rep 18:205–216.

[61] Shimizu, N., Ueno, K., Kurita, E., Shin, SW., Nishihara, T, Amano T, Anzai M, Kishigami S, Kato H, Mitani T, Hosoi Y, et al. (2014) Possible role of ZPAC, zygote-specific proteasome assembly chaperone, during spermatogenesis in the mouse. J Reprod Dev 60:179–186.

[62] Ramos PC, Dohmen RJ (2008) PACemakers of proteasome core particle assembly. Struct Lond Engl 16:1296–1304.

[63] Zuo EW, Yang XG, Lu YQ, Xie L, Shang JH, Li D, Yang H, Hu LL, Zhao HM, Lu SS, et al. (2015) ZPAC is required for normal spermatogenesis in mice. Mol Reprod Dev 82:747–755.

[64] Bosu DR, Kipreos ET (2008) Cullin-RING ubiquitin ligases: global regulation and activation cycles. Cell Div 3:7.

[65] Zhou L, Zhang W, Sun Y, Jia L (2018) Protein neddylation and its alterations in human cancers for targeted therapy. Cell Signal 44:92–102.

[66] Kinterova V, Kanka J, Petruskova V, Toralova T (2019) Inhibition of Skp1-Cullin-F-box complexes during bovine oocyte maturation and preimplantation development leads to delayed development of embryos. Biol Reprod 100:896–906.

[67] Setoyama D, Yamashita M, Sagata N (2007) Mechanism of degradation of CPEB during Xenopus oocyte maturation. Proc Natl Acad Sci USA 104:18001–18006.

[68] Shimuta K, Nakajo N, Uto K, Hayano Y, Okazaki K, Sagata N (2002) Chk1 is activated transiently and targets Cdc25A for degradation at the Xenopus midblastula transition. EMBO J 21:3694–3703.

[69] Kanemori Y, Uto K, Sagata N (2005) Beta-TrCP recognizes a previously undescribed nonphosphorylated destruction motif in Cdc25A and Cdc25B phosphatases. Proc Natl Acad Sci USA 102:6279–6284.

[70] Collart C, Smith JC, Zegerman P (2017) Chk1 Inhibition of the Replication Factor Drf1 Guarantees Cell-Cycle Elongation at the Xenopus laevis Mid-blastula Transition. Dev Cell 42:82-96.e3.

[71] Daldello EM, Le T, Poulhe R, Jessus C, Haccard O, Dupré A (2015) Control of Cdc6 accumulation by Cdk1 and MAPK is essential for completion of oocyte meiotic divisions in Xenopus. J Cell Sci 128:2482–2496.

[72] Ooga M, Suzuki MG, Aoki F (2015) Involvement of histone H2B monoubiquitination in the regulation of mouse preimplantation development. J Reprod Dev 61:179–184.

[73] Jin XL, Chandrakanthan V, Morgan HD, O'Neill C (2009) Preimplantation embryo development in the mouse requires the latency of TRP53 expression, which is induced by a ligand-activated PI3 kinase/AKT/MDM2-mediated signaling pathway. Biol Reprod 80:286–294.

[74] Jones SN, Roe AE, Donehower LA, Bradley A (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 378:206–208.

[75] Montes de Oca Luna R, Wagner DS, Lozano G (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 378:203–206.

[76] Baran V, Brzakova A, Rehak P, Kovarikova V, Solc P (2016) PLK1 regulates spindle formation kinetics and APC/C activation in mouse zygote. Zygote 24:338–345.

[77] Solc P, Kitajima TS, Yoshida S, Brzakova A, Kaido M, Baran V, Mayer A, Samalova P, Motlik J, Ellenberg J (2015) Multiple requirements of PLK1 during mouse oocyte maturation. PloS One 10:e0116783.

[78] Musacchio A, Salmon ED (2007) The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8:379–393.

[79] Roest HP, Baarends WM, de Wit J, van Klaveren JW, Wassenaar E, Hoogerbrugge JW, van Cappellen WA, Hoeijmakers JHJ, Grootegoed JA (2004) The ubiquitin-conjugating DNA repair enzyme HR6A is a maternal factor essential for early embryonic development in mice. Mol Cell Biol 24:5485–5495.

[80] Xu YN, Shen XH, Lee SE, Kwon JS, Kim DJ, Heo YT, Cui XS, Kim NH (2012) Autophagy influences maternal mRNA degradation and apoptosis in porcine parthenotes developing in vitro. J Reprod Dev 58:576–584.

[81] Shen X, Zhang N, Wang Z, Bai G, Zheng Z, Gu Y, Wu Y, Liu H, Zhou D, Lei L (2015) Induction of autophagy improves embryo viability in cloned mouse embryos. Sci Rep 5:17829.

[82] Chi D, Zeng Y, Xu M, Si L, Qu X, Liu H, Li J (2017) LC3-Dependent Autophagy in Pig 2-Cell Cloned Embryos Could Influence the Degradation of Maternal mRNA and the Regulation of Epigenetic Modification. Cell Reprogramming 19:354–362.

[83] Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N (2008) Autophagy is essential for preimplantation development of mouse embryos. Science 321:117–120.

[84] He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43:67–93.

[85] Mizushima N, Komatsu M (2011) Autophagy: renovation of cells and tissues. Cell 147:728–741.

[86] Yue Z, Jin S, Yang C, Levine AJ, Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA 100:15077–15082.

[87] Lee SE, Hwang KC, Sun SC, Xu YN, Kim NH (2011) Modulation of autophagy influences development and apoptosis in mouse embryos developing in vitro. Mol Reprod Dev 78:498–509.

[88] Cho YH, Han KM, Kim D, Lee J, Lee SH, Choi KW, Kim J, Han YM (2014) Autophagy regulates homeostasis of pluripotency-associated proteins in hESCs. Stem Cells Dayt Ohio 32:424–435.

[89] Tsukamoto S, Tatsumi T (2018) Degradation of maternal factors during preimplantation embryonic development. J Reprod Dev 64:217–222.

[90] Yamamoto A, Mizushima N, Tsukamoto S (2014) Fertilization-induced autophagy in mouse embryos is independent of mTORC1. Biol Reprod 91:7.

[91] Laplante M, Sabatini DM (2009) mTOR signaling at a glance. J Cell Sci 122:3589–3594.

[92] Imamura T, Neildez TMA, Thenevin C, Paldi A (2004) Essential role for poly (ADP-ribosyl)ation in mouse preimplantation development. BMC Mol Biol 5:4.

[93] Lee HR, Gupta MK, Kim DH, Hwang JH, Kwon B, Lee HT (2016) Poly(ADP-ribosyl)ation is involved in pro-survival autophagy in porcine blastocysts. Mol Reprod Dev 83:37–49.

[94] Lee JE, Oh HA, Song H, Jun JH, Roh CR, Xie H, Dey SK, Lim HJ (2011) Autophagy Regulates Embryonic Survival During Delayed Implantation. Endocrinology 152:2067–2075.

[95] Tsukamoto S, Hara T, Yamamoto A, Kito S, Minami N, Kubota T, Sato K, Kokubo T (2014) Fluorescence-based visualization of autophagic activity predicts mouse embryo viability. Sci Rep 4:4533.

[96] Lee HR, Kim DH, Kim MG, Lee JS, Hwang JH, Lee HT (2016) The regulation of autophagy in porcine blastocysts: Regulation of PARylation-mediated autophagy via mammalian target of rapamycin complex 1 (mTORC1) signaling. Biochem Biophys Res Commun 473:899–906.

[97] Song BS, Yoon SB, Kim JS, Sim BW, Kim YH, Cha JJ, Choi SA, Min HK., Lee Y, Huh JW, et al. (2012) Induction of autophagy promotes preattachment development of bovine embryos by reducing endoplasmic reticulum stress. Biol Reprod 87:1–11.

[98] Xu YN, Cui XS, Sun SC, Lee SE, Li YH, Kwon JS, Lee SH, Hwang KC, Kim NH (2011) Mitochondrial dysfunction influences apoptosis and autophagy in porcine parthenotes developing in vitro. J Reprod Dev 57:143–150.

[99] Kang MH, Das J, Gurunathan S, Park HW, Song H, Park C, Kim JH (2017) The cytotoxic effects of dimethyl sulfoxide in mouse preimplantation embryos: a mechanistic study. Theranostics 7:4735–4752.

[100] Shin KT, Guo J, Niu YJ, Cui XS (2018) The toxic effect of aflatoxin B1 on early porcine embryonic development. Theriogenology 118:157–163.

[101] Tang L, Yang S, Wang H, Gu H, Xia X, Feng Y, Yang Z, Zhao S, Su C, Su Z, et al. (2018) Nucleoside reverse transcriptase inhibitor-induced rat oocyte dysfunction and low fertility mediated by autophagy. Oncotarget 9:3895–3907.

[102] Lin T, Oqani RK, Lee JE, Kang JW, Kim SY, Cho ES, Jeong YD, Baek JJ, Jin DI (2018) α-Solanine impairs oocyte maturation and quality by inducing autophagy and apoptosis and changing histone modifications in a pig model. Reprod Toxicol Elmsford N 75:96–109.

[103] Sutovsky P, Manandhar G, Laurincik J, Letko J, Caamaño JN, Day BN, Lai L, Prather RS, Sharpe-Timms KL, Zimmer R, et al. (2005) Expression and proteasomal degradation of the major vault protein (MVP) in mammalian oocytes and zygotes. Reprod Camb Engl 129:269–282.

[104] Thrower JS, Hoffman L, Rechsteiner M, Pickart, CM (2000) Recognition of the polyubiquitin proteolytic signal. EMBO J 19:94–102.

[105] Tan JMM, Wong ESP, Kirkpatrick DS, Pletnikova O, Ko HS, Tay SP, Ho MWL, Troncoso J, Gygi SP, Lee MK, et al. (2008) Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Hum Mol Genet 17:431–439.

[106] Sato M, Konuma R, Sato K, Tomura K, Sato K (2014) Fertilization-induced K63-linked ubiquitylation mediates clearance of maternal membrane proteins. Dev Camb Engl 141:1324–1331.

[107] Rojansky R, Cha MY, Chan DC (2016) Elimination of paternal mitochondria in mouse embryos occurs through autophagic degradation dependent on PARKIN and MUL1. eLife 5.

[108] Hajjar C, Sampuda KM, Boyd L (2014) Dual roles for ubiquitination in the processing of sperm organelles after fertilization. BMC Dev Biol 14:6.

[109] Song WH, Yi YJ, Sutovsky M, Meyers S, Sutovsky P (2016) Autophagy and ubiquitin-proteasome system contribute to sperm mitophagy after mammalian fertilization. Proc Natl Acad Sci USA 113:E5261-5270.

[110] Komatsu M, Ichimura Y (2010) Selective autophagy regulates various cellular functions. Genes Cells Devoted Mol Cell Mech 15:923–933.

[111] Benesova V, Kinterova V, Kanka J, Toralova T (2017) Potential Involvement of SCF-Complex in Zygotic Genome Activation During Early Bovine Embryo Development. Methods Mol Biol Clifton NJ 1605:245–257.

[112] Hanson PI, Cashikar A (2012) Multivesicular body morphogenesis. Annu Rev Cell Dev Biol 28:337–362.

[113] Traub LM, Lukacs GL (2007) Decoding ubiquitin sorting signals for clathrin-dependent endocytosis by CLASPs. J Cell Sci 120:543–553.

[114] Sato M, Sato K (2013) Dynamic regulation of autophagy and endocytosis for cell remodeling during early development. Traffic Cph Den 14:479–486.

[115] Sato K, Sato M, Audhya A, Oegema K, Schweinsberg P, Grant BD (2006) Dynamic regulation of caveolin-1 trafficking in the germ line and embryo of Caenorhabditis elegans. Mol Biol Cell 17:3085–3094.

[116] Balklava Z, Pant S, Fares H, Grant BD (2007) Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. Nat Cell Biol 9:1066–1073.

[117] Kadandale P, Stewart-Michaelis A, Gordon S, Rubin J, Klancer R, Schweinsberg P, Grant BD, Singson A (2005) The egg surface LDL receptor repeat-containing proteins EGG-1 and EGG-2 are required for fertilization in Caenorhabditis elegans. Curr Biol CB 15:2222–2229.

[118] Audhya A, McLeod IX, Yates JRIII, Oegema K (2007) MVB-12, a Fourth Subunit of Metazoan ESCRT-I, Functions in Receptor Downregulation. PLoS ONE 2:9.

[119] Zuo Y, Su G, Wang S, Yang L, Liao M, Wei Z, Bai C, Li G (2016) Exploring timing activation of functional pathway based on differential co-expression analysis in preimplantation embryogenesis. Oncotarget 7:74120–74131.

[120] Miller-Fleming L, Olin-Sandoval V, Campbell C, Ralser M (2015) Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell. J Mol Biol 427:3389–3406.

[121] Fenelon JC, Murphy BD (2017) Inhibition of polyamine synthesis causes entry of the mouse blastocyst into embryonic diapause. Biol Reprod 97:119–132.

[122] Lenis YY, Johnson GA, Wang X, Tang WW, Dunlap KA, Satterfield MC, Wu G, Hansen TR, Bazer FW (2018) Functional roles of ornithine decarboxylase and arginine decarboxylase during the peri-implantation period of pregnancy in sheep. J Anim Sci Biotechnol 9:10.

[123] Pendeville H, Carpino N, Marine JC, Takahashi Y, Muller M, Martial JA, Cleveland JL (2001) The ornithine decarboxylase gene is essential for cell survival during early murine development. Mol Cell Biol 21:6549–6558.

[124] Pegg AE (2006) Regulation of ornithine decarboxylase. J Biol Chem 281:14529–14532.

[125] Osborne HB, Duval C, Ghoda L, Omilli F, Bassez T, Coffino P (1991) Expression and post-transcriptional regulation of ornithine decarboxylase during early Xenopus development. Eur J Biochem 202:575–581.

[126] Reverte CG, Ahearn MD, Hake LE (2001) CPEB degradation during Xenopus oocyte maturation requires a PEST domain and the 26S proteasome. Dev Biol 231:447–458.

[127] Mendez R, Barnard D, Richter JD (2002) Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. EMBO J 21:1833–1844.

[128] Hodgman R, Tay J, Mendez R, Richter JD (2001) CPEB phosphorylation and cytoplasmic polyadenylation are catalyzed by the kinase IAK1/Eg2 in maturing mouse oocytes. Dev Camb Engl 128:2815–2822.

[129] Uzbekova S, Arlot-Bonnemains Y, Dupont J, Dalbiès-Tran R, Papillier P, Pennetier S, Thélie A, Perreau C, Mermillod P, Prigent C, et al. (2008) Spatio-temporal expression patterns of aurora kinases a, B, and C and cytoplasmic polyadenylation-element-binding protein in bovine oocytes during meiotic maturation. Biol Reprod 78:218–233.

[130] Bowerman B, Kurz T (2006) Degrade to create: developmental requirements for ubiquitin-mediated proteolysis during early C. elegans embryogenesis. Dev Camb Engl 133:773–784.

[149] Lu X, Gao Z, Qin D, Li L (2017) A Maternal Functional Module in the Mammalian Oocyte-To-Embryo Transition. Trends Mol Med 23:1014–1023.

[150] Bebbere D, Masala L, Albertini DF, Ledda S (2016) The subcortical maternal complex: multiple functions for one biological structure? J Assist Reprod Genet 33:1431–1438.

[151] Duesbery NS, Choi T, Brown KD, Wood KW, Resau J, Fukasawa K, Cleveland DW, Vande Woude GF (1997) CENP-E is an essential kinetochore motor in maturing oocytes and is masked during mos-dependent, cell cycle arrest at metaphase II. Proc Natl Acad Sci USA 94:9165–9170.

[152] Allard P, Champigny MJ, Skoggard S, Erkmann JA, Whitfield ML, Marzluff WF, Clarke HJ (2002) Stem-loop binding protein accumulates during oocyte maturation and is not cell-cycle-regulated in the early mouse embryo. J Cell Sci 115:4577–4586.

[153] Toralova T, Susor A, Nemcova L, Kepkova K, Kanka J (2009) Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. Reprod Camb Engl 138:o. 5, pp. 783–791, Nov. 2009.

[154] Collart C, Allen GE, Bradshaw CR, Smith JC, Zegerman P (2013) Titration of four replication factors is essential for the Xenopus laevis midblastula transition. Science 341:893–896.

[155] Fisher D (2011) Control of DNA replication by cyclin-dependent kinases in development. Results Probl Cell Differ 53:201–217.

[156] Ichikawa K, Noda T, Furuichi Y (2002) Preparation of the gene targeted knockout mice for human premature aging diseases, Werner syndrome, and Rothmund-Thomson syndrome caused by the mutation of DNA helicases. Nihon Yakurigaku Zasshi Folia Pharmacol Jpn 119:219–226.

[157] Hoki Y, Araki R, Fujimori A, Ohhata T, Koseki H, Fukumura R, Nakamura M, Takahashi H, Noda Y, Kito S, et al. (2003) Growth retardation and skin abnormalities of the Recql4-deficient mouse. Hum Mol Genet 12:2293–2299.

[158] Wu J, Capp C, Feng L, Hsieh T (2008) Drosophila homologue of the Rothmund-Thomson syndrome gene: essential function in DNA replication during development. Dev Biol 323:130–142.

[159] Jeon Y, Ko E, Lee KY, Ko MJ, Park SY, Kang J, Jeon CH, Lee H, Hwang DS (2011) TopBP1 deficiency causes an early embryonic lethality and induces cellular senescence in primary cells. J Biol Chem 286:5414–5422.

[160] Sansam CL, Cruz NM, Danielian PS, Amsterdam A, Lau ML, Hopkins N, Lees JA (2010) A vertebrate gene, ticrr, is an essential checkpoint and replication regulator. Genes Dev 24:183–194.

[161] Yao L, Chen J, Wu X, Jia S, Meng A (2017) Zebrafish cdc6 hypomorphic mutation causes Meier-Gorlin syndrome-like phenotype. Hum Mol Genet 26:4168–4180.

[162] El Dika M, Laskowska-Kaszub K, Koryto M, Dudka D, Prigent C, Tassan JP, Kloc M, Polanski Z, Borsuk E, Kubiak JZ (2014) CDC6 controls dynamics of the first embryonic M-phase entry and progression via CDK1 inhibition. Dev Biol 396:67–80.

[163] Zhou ZW, Liu C, Li TL, Bruhn C, Krueger A, Min WK, Wang ZQ, Carr AM (2013) An essential function for the ATR-activation-domain (AAD) of TopBP1 in mouse development and cellular senescence. PLoS Genet 9:e1003702.

[164] Eisenmann KM, West RA, Hildebrand D, Kitchen SM, Peng J, Sigler R, Zhang J, Siminovitch KA, Alberts AS (2007) T cell responses in mammalian diaphanous-related formin mDia1 knock-out mice. J Biol Chem 282:25152–25158.

[165] Peng J, Wallar BJ, Flanders A, Swiatek PJ, Alberts AS (2003) Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. Curr Biol CB 13:534–545.

[166] Cheng Y, Quinn JF, Weiss LA (2013) An eQTL mapping approach reveals that rare variants in the SEMA5A regulatory network impact autism risk. Hum Mol Genet 22:2960–2972.

[167] Silva T, Bradley RH, Gao Y, Coue M (2006) Xenopus CDC7/DRF1 complex is required for the initiation of DNA replication. J Biol Chem 281:11569–11576.

[168] Takahashi TS, Walter JC (2005) Cdc7-Drf1 is a developmentally regulated protein kinase required for the initiation of vertebrate DNA replication. Genes Dev 19:2295–2300.

[169] Tikhmyanova N, Coleman TR (2003) Isoform switching of Cdc6 contributes to developmental cell cycle remodeling. Dev Biol 260:362–375.

[170] Solc P, Saskova A, Baran V, Kubelka M, Schultz RM, Motlik J (2008) CDC25A phosphatase controls meiosis I progression in mouse oocytes. Dev Biol 317:260–269.

[171] Farrell JA, Shermoen AW, Yuan K, O'Farrell PH (2012) Embryonic onset of late replication requires Cdc25 down-regulation. Genes Dev 26:714–725.

[172] Sibon OC, Stevenson VA, Theurkauf WE (1997) DNA-replication checkpoint control at the Drosophila midblastula transition. Nature 388:93–97.

Figure Legends

Figure 1. The comparison of development of selected mammalian and non-mammalian species. The figure shows the most important events occurring during the early development of Xenopus, Drosophila, mouse, bovine and human embryos compared to each other in the timeline. **A)** Hpf/hped vs. developmental stage; **B)** Hpf/hped vs. event; **C)** Stage vs. event

2c - two-cell stage embryos; 4c - four-cell stage embryos; e8c – early eight-cell stage embryos; L8c – late eight-cell stage embryos; hpf – hours post fertilization; hped – hours post egg deposition; EGA – embryonic genome activation; MBT – mid-blastula transition

Tables

Protein	Function	Organism	Reference
TAB1	Regulator of MAP3K7/TAK1, NFKB, MAPK14/p38alpha	mouse	[60]
Cdc25A	Induces progression of mitosis; tyrosine protein phosphatase	Xenopus	[68, 69]
Cdc6	Controls progression of DNA replication	Xenopus	[8]
Treslin	Regulates DNA replication	Xenopus	[8]
RecQL4	DNA dependent ATPase	Xenopus	[8]
TopBp1	Involved in DNA replication	Xenopus	[8]
Drf1/DBF4B	Plays crucial role DNA replication control, Cdc7 subunit	Xenopus	[154]

Table 1: Proteins degraded in the time of major EGA. The degradation of these proteins in necessary for normal course of EGA.

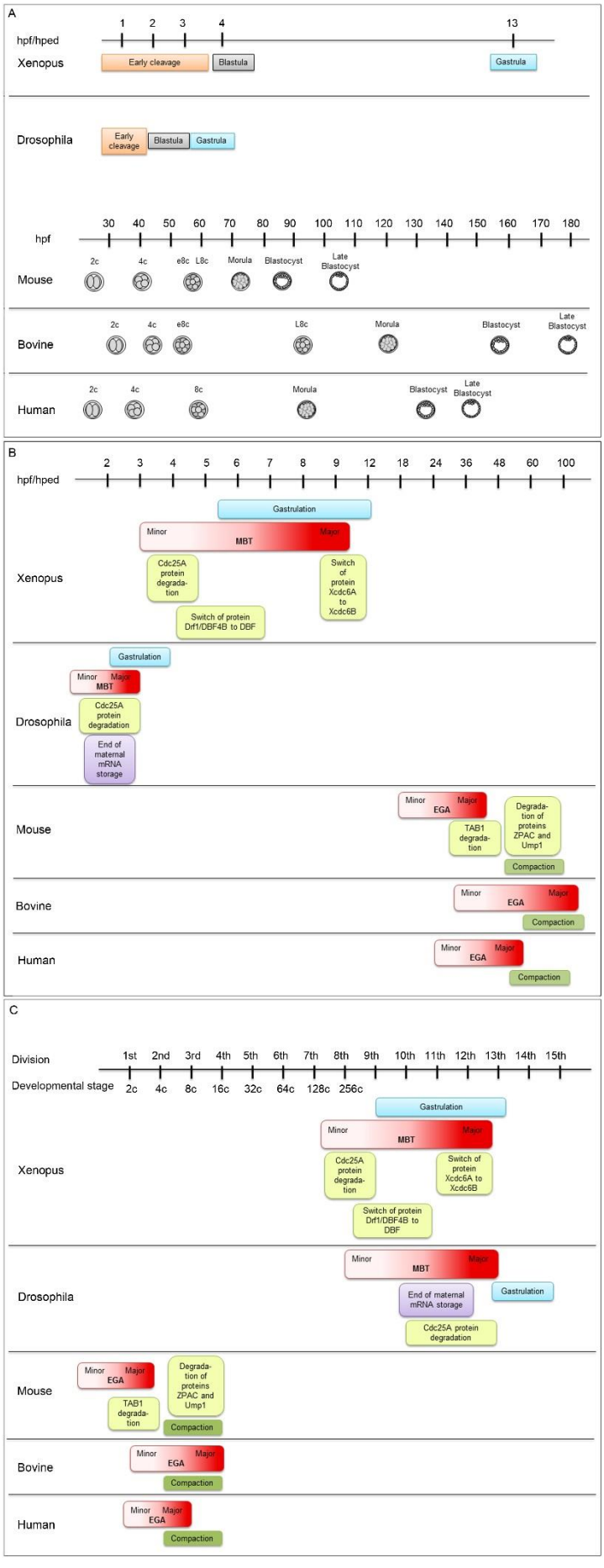

A
hpf/hped
1 2 3 4 13
Xenopus
Early cleavage
Blastula
Gastrula
Drosophila
Early cleavage
Blastula
Gastrula
hpf
30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Mouse
2c 4c e8c L8c Morula Blastocyst Late Blastocyst
Bovine
2c 4c e8c L8c Morula Blastocyst Late Blastocyst
Human
2c 4c 8c Morula Blastocyst Late Blastocyst
B
hpf/hped
2 3 4 5 6 7 8 9 12 18 24 36 48 60 100
Xenopus
Gastrulation
Minor MBT Major
Cdc25A protein degrada-tion
Switch of protein Drf1/DBF4B to DBF
Switch of protein Xcdc6A to Xcdc6B
Drosophila
Gastrulation
Minor MBT Major
Cdc25A protein degradation
End of maternal mRNA storage
Mouse
Minor EGA Major
TAB1 degrada-tion
Degrada-tion of proteins ZPAC and Ump1
Compaction
Bovine
Minor EGA Major
Compaction
Human
Minor EGA Major
Compaction
C
Division
1st 2nd 3rd 4th 5th 6th 7th 8th 9th 10th 11th 12th 13th 14th 15th
Developmental stage
2c 4c 8c 16c 32c 64c 128c 256c
Xenopus
Gastrulation
Minor MBT Major
Cdc25A protein degrada-tion
Switch of protein Xcdc6A to Xcdc6B
Switch of protein Drf1/DBF4B to DBF
Drosophila
Minor MBT Major
End of maternal mRNA storage
Gastrulation
Cdc25A protein degradation
Mouse
Minor EGA Major
TAB1 degrada-tion
Degrada-tion of proteins ZPAC and Ump1
Compaction
Bovine
Minor EGA Major
Compaction
Human
Minor EGA Major
Compaction

Figure 1

5. Sumární diskuze

Během časného preimplantační vývoje savců probíhá celá řada klíčových dějů, které mají za výsledek přeměnu vysoce specializovaných gamet- spermie a oocytu, v totipotentní embryo, které je schopné vytvořit úplně nový organizmus. Počáteční část vývoje je řízena maternální mRNA a proteiny, které byly nasyntetizovány a uloženy v oocytu již z období oogeneze. Tyto zdroje embryo využívá až do chvíle, dokud nedojde k aktivaci embryonálního genomu (EGA), kdy začíná transkripce z genomu embrya. Všechny počáteční procesy savčího embryonálního vývoje jsou pod maternální kontrolou, patří mezi ně oplození, blok proti polyspermii, zpracování paternální DNA, udržení homeostáze a první kroky embryonálního vývoje. Bylo zjištěno, že po oplození dochází k postupné degradaci maternálních rezerv. Pravděpodobně se jedná o postupný přechod kontroly z uložených maternálních mRNA a proteinů k nově aktivovanému embryonálnímu genomu sérií sehraných kroků. Jde tedy o více jednotlivých podprocesů, než náhlé radikální změny (Li et al. 2013).

Maternální mRNA jsou z embrya odstraněny pomocí mikroRNA (miRNA) a jejich degradace je pro následující embryonální vývoj myši nezbytná (Tripurani et al. 2013). Většina myších mRNA je destabilizovaná již před EGA (Su et al. 2007), přičemž v 2buněčném stádiu můžeme detekovat pouze 10 – 20 % původní maternální mRNA (Yokoi et al. 1993). O degradaci maternálních proteinů však není doposud příliš mnoho informací. Předpokládá se, že i degradace maternálních proteinů je pro další embryonální vývoj nezbytná, nicméně přesný mechanizmus ještě není znám. Tento proces však pravděpodobně nebude tak rychlý, jako degradace maternálních mRNA a některé proteiny mohou setrvávat i po EGA. Li et al. (2013) uvádí, že některé maternální proteiny mohou tvořit komplexy, díky čemuž mohou přetrvat i do pozdějších stádií vývoje. Mezi tyto proteiny patří například proteiny *zony pellucidy* (ZPs), bez nichž by embryo v počátku vývoje zaniklo, nebo subkortikální maternální komplex (SCMC) (Li et al. 2013). Tyto proteiny jsou v embryu uloženy až do stádia blastocysty, přestože je jejich mRNA degradována již v průběhu EGA (Bebbere et al. 2016). Další proteiny mohou být před degradací chráněny pomocí maskování, případně jinak modifikovány (Li et al. 2013). Jiné maternální proteiny však musí být z embrya odstraněny. Mezi nejpravděpodobnější způsoby degradace patří ubiquitin-proteazomový systém. Tomu nasvědčuje i fakt, že krátce po oplození je možné u myších zygot detekovat vysoké množství proteinů náležících právě do ubiquitinační dráhy, což má pravděpodobně za následek velký úbytek proteinů mezi myšími MII oocyty a zygotami (Li et al. 2013). Cílem mé práce bylo objasnění funkce SCF komplexu, jedné

z nejvýznamnějších E3 ubiquitin-ligáz, v průběhu preimplantačního vývoje. Jeho funkce je důležitá pro transkripci, gametogenezi, replikaci DNA a kontrolu buněčného cyklu (Petroski & Deshaies 2005), jeho význam při časné embryogenezi však nebyl doposud objasněn. Nejprve jsem se zaměřila na expresi jednotlivých členů SCF komplexu, jejich lokalizaci v rámci jednotlivých vývojových stádií a přítomnost aktivního SCF komplexu v průběhu časného preimplantačního vývoje. Dynamika exprese mRNA mezi jednotlivými vývojovými stádii koresponduje s důležitostí daného genu pro vývoj, přičemž geny aktivované do hlavní vlny EGA jsou považovány za nejdůležitější. Umlčení těchto genů může vést k snížení kvality vývoje nebo dokonce k jeho úplnému zastavení (Falco et al. 2007; Toralová et al. 2009; Salilew-Wondim et al. 2010). Geny spjaté s ubiquitinací jsou převážně aktivovány v 8buněčném stádiu (Graf et al. 2014), nicméně naše výsledky prokázaly ještě dřívější aktivaci některých členů SCF komplexu. Konkrétně Cul1 a Skp1 se transkribuje již od 4buněčného a časného 8buněčného stádia. Transkripce Rbx1 začíná později, během hlavní vlny EGA. Z toho vyplývá, že jednotliví členové SCF komplexu mají pro správnou aktivaci embryonálního komplexu nepopiratelnou roli. Zejména velmi časný počátek exprese Cul1, který byl detekován i u *Drosophily* (Filippov et al. 2000), dělá z Cul1 nejdůležitějšího člena SCF komplexu. Toto je podpořeno i existencí dvou variant Cul1, které jsou transkribovány ze dvou rozdílných genů (Kepkova et al. 2011). Embryonální verze Cul1 je transkribována od 4buněčného stádia. V tomto stádiu je maternální verze Cul1 ještě detekovatelná, v pozdějších stádiích (od časného 8buněčného stádia) však maternální forma Cul1 není téměř znatelná a tudíž od tohoto okamžiku je embryo plně závislé pouze na embryonální verzi.

Dále jsme sledovali vazbu mezi CUL1 a SKP1, která signalizuje aktivaci celého SCF komplexu. K tomu jsme využili Duolink *in-situ* PLA metodu, která je schopná detekovat signál v případě, že se dva hledané proteiny pohybují ve vzdálenosti maximálně 40 nm, tedy pokud interagují. Pozitivní PLA signál byl objeven u všech vývojových stádiích v podobné intenzitě. Nejvyšší aktivita byla detekována u MII oocytů, kde je náležitá degradace proteinů nezbytně důležitá pro správné zrání oocytu, nicméně ani tento rozdíl v intenzitě signálu nebyl statisticky významný. Za nejdůležitější ubiquitin-ligázu pro meiotické zrání se považuje APC/C (Karabinova et al. 2011). Bylo však zjištěno, že degradace mitotického APC/C je kontrolována pomocí SCF komplexu (Guardavaccaro et al. 2003), což vede k hypotéze, že SCF komplex by mohl hrát podobnou roli i v průběhu meiózе. Pomocí PLA analýzy u embryí ve stádiu blastocysty byl objeven velký nepoměr v přítomnosti signálu mezi ICM a TE blastomerami. Téměř žádný signál nebyl objeven v ICM, zatímco v TE blastomerách byl signál velmi intenzivní. S tímto objevem se shodují i výsledky (Sutovsky et al. 2001), kteří prokázali, že

v oblasti TE dochází k radikální proteolytické degradaci pomocí ubiquitinu mající vliv na pozdější formování extraembryonální tkáně. Vyšší aktivitu SCF komplexu v trofoektodermu podporují také naše výsledky, kdy CUL1 byl ve stádiu blastocysty detekován obzvláště v jádrech a také především v oblasti TE. To je v souladu s výsledky (Lee et al. 2003), kteří prokázali důležitou roli Cul1 pro tvorbu trofoektodermu a placenty. Překvapivě však rozdíly v aktivitě SCF komplexu nebyly potvrzeny množstvím proteinu žádného z našich tří zkoumaných genů. Cullin1 je považován za limitní faktor pro aktivitu SCF komplexu (Liu et al. 2002), nicméně hladina CUL1 postupně rostla od MII oocytu do pozdního 8buněčného stádia a následně zůstala stabilní do stádia moruly. PLA signál, který byl nejvyšší u oocytů v MII stádiu, následně znatelně (avšak nesignifikantně) klesl v 4buněčném stádiu a zůstal stejný do stádia moruly. Výsledky lokalizace proteinů, PLA signál ve stádiu blastocysty, změna hladiny proteinů a aktivita SCF komplexu naznačuje, že všechny tři základní komponenty SCF komplexu mohou mít i další funkce nezávislé na SCF komplexu. To potvrzuje i fakt, že Rbx1 může interagovat i s ostatními členy rodiny cullinů a tak může být i součástí jiných komplexů (Piva et al. 2002). Exprese proteinu i mRNA Rbx1 nesouhlasí expresním profilům dalších dvou neměnných členů SCF komplexu- Cul1 a Skp1. Rbx1 je pro funkčnost SCF komplexu nezbytný a po jeho umlčení je celý komplex neaktivní (Zhou et al. 2013), exprese proteinu RBX1 nekoreluje s aktivitou SCF komplexu. To částečně platí i pro zbývající dva proteiny, i přes to, že všechny dosud známé funkce Cul1 jsou spjaty s SCF komplexem. Skp1 zastává i jiné funkce (mimo SCF komplex), ale ty byly prozatím objeveny jen u nižších organismů (Zhang et al. 1995). Této teorii nasvědčuje i existence komplexů Skp1, které se objevují jako vyšší bandy po western blot analýze.

Profily exprese členů SCF komplexu, existence dvou forem Cul1 a aktivita SCF komplexu během celého preimplantačního vývoje ukazuje nezbytnost SCF komplexu pro preimplantační vývoj skotu. Z toho důvodu jsme se rozhodli pokračovat ve studiu této problematiky a rozšířit základní povědomí o roli SCF komplexu v časné embryogenezi o další poznatky. Dalším krokem bylo umlčení SCF komplexu. K tomu jsme využili inhibitor MLN4924 (také známý jako Pevonedistat), který inhibuje SCF ligázy řízené neddylací. Mezi ně patří primárně SCF ligázy obsahující Cul1, nebo v menším měřítku další komplexy obsahující zbývající culliny (Cul2, Cul3, Cul4 nebo Cul5). Inhibitor MLN4924 se primárně používá jako lék proti rakovině, tudíž je většina výzkumů jeho účinku soustředěná na rakovinné buňky (Zhou et al. 2018). První volbou pro umlčení SCF komplexu bylo použití RNA interference (čímž by mělo dojít k inaktivaci celého SCF komplexu), tedy mikroinjikace dsRNA do zygot. Ačkoliv je tato metoda pro dané účely běžně využívána, po umlčení mRNA

Cul1, zůstával protein CUL1 stabilní a přítomný bez jakékoliv změny. Z toho důvodu byla zvolena inhibice pomocí MLN4924 jako vhodnější alternativa.

Nejprve jsme zjišťovali, jaký dopad bude mít deaktivace SCF komplexů na zrající oocyty. Oocyty byly kultivovány v MLN4924 od GV stádia po stádium MII. Již během tohoto období byly pozorovány očividné změny v morfologii kumulárních buněk. Zatímco u kontrolních oocytů byly kumulární buňky expandované a po oplození následně začaly ze zygot opadávat, u inhibovaných oocytů nebyl podobný průběh zaznamenán. I přes vliv kultivace v inhibitoru na expanzi kumulu se však neprojevil žádný statisticky významný nárůst v obsahu celkového proteinu u buněk kumulu oproti kontrolám. Role ubiquitin-proteazomového systému v expanzi kumulu ještě není příliš objasněna. Yi et al. (2008) však uvádí, že po kultivaci oocytů v inhibitoru MG132 po dobu 22 hodin, žádný oocyt nedosáhl MII stádia. Na rozdíl od inhibitoru MG132, který inhibuje aktivitu celého proteazomu, MLN4924 inhibuje pouze aktivaci SCF komplexů. To je pravděpodobně důvodem, proč téměř 74% oocytů ošetřených MLN4924 mohlo být oplozeno. Úspěšný vznik diploidních zygot však bylo zaznamenáno jen u 40,3 % případů, na rozdíl od 80 % diploidních kontrolních zygot. Předpokládáme, že vyšší procento polyspermie bylo výsledkem nefunkční exocytózy kortikálních granulí. Jejich exocytózu zajišťují proteiny vrstvy *zona pellucida* a jejich absence způsobená inhibicí deubiquitinačních enzymů (DUB) UCHL1 způsobuje nárůst v polyspermii (Susor et al. 2010). To naznačuje, že degradace pomocí SCF ligáz při ochraně proti polyspermii kooperuje s UCHL1 nebo dalšími DUB enzymy.

Když jsme sledovali vývojovou kompetenci embryí vzniklých z oocytů kultivovaných v MLN4924, nalezli jsme statisticky významně opoždění vývoj v porovnání s kontrolami. Nejvíce signifikantní rozdíl byl patrný u embryí v 8buněčných stádiích a síla významnosti v dalších stádiích klesala. Podobný výsledek měla i kultivace embryí v MLN4924 od 4buněčného do pozdního 8buněčného stádia, kde jsme nalezli významné rozdíly u embryí ve stádiu moruly a blastocysty (168 hpf) v porovnání s kontrolami. Pozdější kultivací byly však statisticky významné rozdíly snižovány, a přestože u blastocyst 192hpf byly rozdíly ještě patrné, již nebyly signifikantní. To naznačuje, že některá embrya jsou schopná překonat nevýhodu v podobě kultivace v MLN4924 a vyvinout se v blastocysty o 24 hodin později. Předpokládáme, že embrya byla zastavena ve svém vývoji v 8buněčném stádiu a po přenosu do čistého, MLN4924 neobsahujícího média byla schopna po pár hodinách ve svém vývoji pokračovat. Z našich výsledků vyplývá, že aktivita SCF komplexu je více důležitá pro zrání oocytů než během časné embryogeneze, když po kultivaci GV-MII oocytů v MLN4924 byl následující embryonální vývoj horší, než při inhibici až od 4 do pozdních 8buněčných stádií.

Zpožděný vývoj embryí může být daný redukcí hladiny množství mRNA EGA markerů PAPOLA a U2AF1A. Hladina markeru eiF1A nebyla signifikantně změněna. Geny, které se podílí na ubiquitinaci proteinů jsou u skotu aktivovány v 8buněčném stádiu (Graf et al. 2014), tedy ještě před hlavní vlnou EGA. Opožděná aktivace embryonálního genomu se projevila také u myších embryí kultivovaných s MG132 (Shin et al. 2010). Náš výzkum prokázal také opožděnou EGA a naznačuje tak, že degradace pomocí SCF komplexu je pro preimplantační vývoj důležitá. V průběhu našich experimentů však nedocházelo k opoždění aktivace eiF1A, což se s výsledky Shin et al. (2010) rozchází. Vyplývá z toho, že pro normální funkci EGA je potřebná kooperace více částí ubiquitin-proteozomového systému.

Přestože jsme neprokázali signifikantní nárůst celkového množství proteinů u kumulárních buněk, nalezli jsme statisticky významný nárůst v množství proteinů u inhibovaných oocytů i embryí. Z toho vyplývá, že po inhibici SCF komplexu nedochází k degradaci některých proteinů a ty jsou následně v oocytu/embryu hromaděny. Testovali jsme několik substrátů SCF komplexu (SMAD4, RPS6, CENPE, IκBA, P27) (Yaron et al. 1998; Tsvetkov et al. 1999; Wan et al. 2004; Liu et al. 2006; Xiao et al. 2015) a další proteiny na základě jejich exprese v průběhu preimplantačního vývoje (RBM19, ZAR1) (Pennetier et al. 2004; Bebbere et al. 2008; Zhang et al. 2008a) pomocí metody western blot. V žádným z uvedených proteinů se však nepovedl prokázat statisticky významný nárůst. Zhang et al. (2015) a Tong et al. (2017) prokázali hromadění proteinu P27 po použití inhibitoru MLN4924 na buněčné kultury. Yang et al. (2017) po inhibici E3 ligázy RNF114 u myších embryí objevili nárůst pouze jednoho proteinu- TAB1 (TGF-beta activated kinase 1), přestože testovali přes 9000 proteinů. To naznačuje, že se degradace proteinů během maturace oocytů a preimplantačního vývoje řídí striktními pravidly. U preimplantačních embryí je nutné, aby maternální rezervy mRNA a proteinů vydržely alespoň do stádia, ve kterém dochází k aktivaci embryonálního genomu. Proto předpokládáme, že SCF komplexy by mohly být zapojeny do degradace zmiňovaných proteinů, ale jejich degradace by mohla být omezená pouze na specifický čas nebo místo, podobně jako existence translačních hotspotů během maturace oocytů (Susor et al. 2015). Překvapivě jsme objevili signifikantní snížení proteinu SMAD4 u MII oocytů inhibovaných MLN4924 v porovnání s kontrolami. Předpokládáme, že tento pokles může být spjat s atypickou maturací ošetřených oocytů a kumulárních buněk, jelikož knockout Smad4 genu je spojený s poškozením kumulárních buněk myši (Pangas et al. 2006).

Během testování jednotlivých proteinů metodou western blot jsme narazili na zajímavý fenomén. Jak bylo zmiňováno již při hodnocení výsledků exprese proteinu SKP1, u dalších dvou zmiňovaných proteinů (P27 a IκBA) jsme objevili bandy s vyšší molekulární hmotností,

než bylo očekáváno. Tyto bandy nebylo možné rozpustit použitím dithiothreitolu nebo vyšší teploty (var po dobu 5 min). Pro kontrolu byly použity buňky fibroblastů, u kterých se žádné vyšší bandy nepotvrdily. To naznačilo, že bandy s vyšší molekulární hmotností by mohly být specifické pro preimplantační embrya. Je známo, že proteiny embryí mohou mít rozdílné izoformy (Tay et al. 2006), nicméně velké rozdíly v molekulární hmotnosti toto vysvětlení nepotvrdily. Zároveň nešlo o polymerizaci, jelikož velikost bandů neodpovídala násobkům předpokládaných hodnot. Předpokládáme, že tyto bandy by mohly být komplexy proteinů, které vznikly, aby je ochránily před předčasnou degradací pro vyšší vývojová stádia (pravděpodobně postimplantační). Domníváme se, že podobné výsledky by mohly být potvrzeny i u jiných proteinů, zbývá však určit, jaký význam tyto bandy s vyšší molekulární hmotností mají.

Analýza exprese jednotlivých členů SCF komplexu prokázala jejich nezbytnost pro normální preimplantační vývoj. Zejména časná aktivace Cul1 a Skp1 naznačila jejich nepostradatelnost pro správnou EGA. Ubiquitinace zprostředkovaná SCF komplexem je v přibližně stejném zastoupení v průběhu celého preimplantačního vývoje. Ve stádiu blastocysty se však jeho aktivita koncentruje do trofoektodermu. Srovnání množství jednotlivých proteinů a aktivity SCF komplexu spolu vzájemně nesouhlasí. V MII stádiu jsou proteiny exprimovány na nejnižší hladině, ale SCF komplex je v tomto stádiu nejaktivnější. To však neznamená, že úroveň exprese proteinu neovlivňuje SCF komplexem zprostředkovanou ubiquitinaci, jak prokázal již Piva et al. (2002).

Prokázali jsme, že SCF ligázy jsou pro normální maturaci oocytů, expanzi kumulárních buněk a správný preimplantační vývoj embryí skotu nezbytné. Přestože jsme našli nárůst v celkovém množství proteinů u oocytů i embryí s inhibovanými SCF ligázami, neobjevili jsme žádný protein, který by byl tímto nárůstem ovlivněn. Na zjištění, které proteiny se u embryí hromadí, bude v následujících pokusech použita hmotnostní spektrometrie. To však bude vyžadovat nashromáždění velkého množství inhibovaných oocytů a embryí, což může být vzhledem k obtížnosti získávání bovinního materiálu problém. Je to pravděpodobně však jediná cesta, jak objevit, o které proteiny se jedná.

Ze všech dostupných informací o degradaci proteinů v průběhu časného preimplantačního vývoje jsme sepsali review. V něm shrnujeme dosud známé poznatky o odstraňování proteinů v průběhu embryogeneze savců a mezery vyplňujeme vědomostmi zjištěnými u nižších živočichů. Tato studie bude fungovat jako příprava pro vypracování teoretického modelu, který by měl ulehčit pochopení dějů spjatých s degradací proteinů u savčích embryí a usnadnit přenos vědomostí mezi jednotlivými modelovými organizmy. Pochopení mechanizmů degradace maternálních proteinů, které jsou jednou z nezbytných

podmínek úspěšné aktivace embryonálního genomu, je nezbytné pro následující vývoj a vylepšení technik *in vitro* oplození a kultivace embryí hospodářských zvířat. Ze znalostí získaných na modelových organizmech bude profitovat i humánní asistovaná reprodukce.

6. Závěr

V této práci jsme sledovali význam SCF komplexu v průběhu preimplantačního vývoje skotu. Popsali jsme expresi a lokalizaci jeho jednotlivých členů a následně i lokalizaci aktivního SCF komplexu. Podařilo se prokázat, že především u Cul1 a Skp1 dochází k velmi časné aktivaci embryonální transkripce, což naznačuje jejich nezbytnost pro správnou aktivaci embryonálního genomu. Proto jsme předpokládali, že se SCF komplex účastní v degradaci maternálních proteinů a abychom tuto skutečnost ověřili, SCF komplex jsme inhibovali. Kultivací oocytů a embryí v médiu s inhibitorem MLN4924, jsme zabránili degradaci proteinů řízené neddylací, což zahrnuje degradaci řízenou SCF ligázami. Výsledky našich studií ukázaly, že degradace pomocí SCF ligáz je nezbytným předpokladem pro normální zrání oocytů, expanzi kumulárních buněk i správný preimplantační vývoj skotu. Zvláště inhibice SCF ligáz u oocytů měla velký dopad na následné *in vitro* oplození a embryogenezi, kdy se projevil zhoršený preimplantační vývoj od 4buněčného stádia. Inhibice SCF ligáz zároveň ovlivnila i aktivaci embryonálního genomu, kdy některé markery EGA (U2AF1 a PAPOLA) projevili opožděnou aktivaci z embryonálního genomu. Oocyty i embrya s umlčenými SCF ligázami projevila signifikantní nárůst v celkovém množství proteinů. Tyto výsledky částečně potvrzují naší hypotézu, že degradace přinejmenším některých maternálních proteinů je pro EGA nezbytná. Prozatím se však nepodařilo prokázat, o které konkrétní proteiny se jedná. To by měl odhalit náš následující výzkum, ve kterém budeme analyzovat inhibované oocyty a embrya pomocí hmotnostní spektrometrie. Na základně našeho review bude následně sestaven matematický model. Díky němu budeme schopni přenést vědomosti z nižších organizmů i na savce a bude tak snazší pochopit, jak probíhá degradace maternálních proteinů i u savčích embryí.

7. Seznam zkratek

APC/C	anafázi podporující komplex/cyklozom (Anaphase-Promoting Complex or Cyclosome)
Atg	protein související s autofagií (Autophagy-Related Protein)
ATP	adenosin trifosfát
Ca2+	vápenaté kationty
Cand1	Cullin-Associated and Neddylation Dissociated Protein 1
Cdc25A	Cell Division Cycle 25A
Cdc6	Cell Division Cycle 6
CENPE	Centromere-Associated Protein E
COP9/signalazom	Constitutive Photomorphogenesis 9/Signalosome
Cul	cullin
Drf1/DBF4B	DBF4 Zinc Finger B
dsRNA	dvouvláknová ribonukleová kyselina (double-stranded RNA)
DUBs	deubiquitinační enzymy
E1 enzym	ubiquitin aktivující enzym
E2 enzym	ubiquitin konjugující enzym
E3 enzym	ubiquitin ligáza
EGA	aktivace embryonálního genomu (embryonic genome activation)
eiF1A	eukaryotický translační iniciační faktor 1A (Eukaryotic Translation Initiation Factor 1A)
eIF4G	eukaryotický translační iniciační faktor 4 gama (Eukaryotic Translation Initiation Factor 4 Gamma)
FBXO30	F-box protein 30
G1 fáze	růstová fáze 2 buněčného cyklu (gap phase 1)
G2 fáze	růstová fáze 1 buněčného cyklu (gap phase 2)
GV	oocyt ve stádiu zárodečného váčku (germinal vesicle)
HECT	ubiquitin ligázy obsahující motiv HECT (Homologous to E6-Associated Protein C-terminus)
Hpf	hodiny po oplození (hours post fertilization)
ICM	vnitřní buněčná masa (inner cell mass), embryoblast
IP3	inozitoltrisfosfát

IκB	inhibitor kappa B (Inhibitor of Kappa B)
Lys-63,48,29	lysin 63, 48, 29
MCL1	Induced Myeloid Leukemia Cell Differentiation Protein
MG132	inhibitor proteazomu
MII	metafáze druhého meiotického dělení
miRNA	mikroRNA
MLN4924	inhibitor neddylace proteinů, Pevonedistat
mRNA	messengerová jednovláknová ribonukleová kyselina
mTOR	cílový protein rapamycinu (Mammalian Target of Rapamycin)
MUL1	Mitochondrial E3 ubiquitin protein ligase 1
MZT	přechod z maternální k embryonální kontrole vývoje
N:C poměr	nukleocytoplazmatický poměr (N:C ratio)
Na/K ATPáza	sodno-draselná ATPázová pumpa
Nanog	transkripční faktor Nanog
Nedd8	ubiquitinu podobný protein Nedd8 (Ubiquitin-Like Protein Nedd8)
Oct4	oktamer vázající transkripční faktor 4 (Octamer-Binding Transcription Factor 4)
P21	protein p21, inhibitor kinázy závislé na cyklinu (cyclin-dependent kinase inhibitor 1A)
P27	protein p27, inhibitor kinázy závislé na cyklinu (Cyclin-dependent kinase inhibitor 1B)
P53	Tumor protein p53
PAPOLA	Poly(A) Polymerase Alpha
PARKIN	Parkinson protein
PIP2	fosfatidylinositol-4,5-bisfosfát
PLA	*in situ* Proximity Ligation Assay
PLCζ	fosfolipáza C zeta
RBM19	RNA Binding Motif Protein 19
Rbx1	RING box protein-1
RecQL4	RecQ Like Helicase 4
RING	ubiquitin ligázy obsahující RING finger doménu (Really Interesting New Gene)
RNF114	Ring finger protein 114

RNF114	E3 ubiquitin-ligáza RNF114
ROC1	regulátor cullinů (Regulator Of Cullins)
RPS6	ribozomální protein S6 (Ribosomal Protein S6)
RT-PCR	reverzně transkripční polymerázová řetězová reakce
SCF ^{Fbw7}	SCF komplex obsahující Fbw7 (F-Box And WD Repeat Domain Containing 7)
SCF komplex	Skp1-Cul1-F box komplex
$SCF^{\beta TrC}$	SCF komplex obsahující βTrC
SCMC	subkortikální maternální komplex, subcortical maternal complex
Skp1	S-Phase Kinase-Associated Protein 1
SLBP	Stem-loop vazebný protein (Stem-Loop-Binding Protein)
SMAD4	Mothers Against Decapentaplegic Homolog 4
Sox2	transkripční faktor Sox2
TAB1	TGF-beta activated kinase 1
TAB1	TGF-beta activated kinase 1 binding protein
TE	trofoektoderm
U2AF1	U2 Small Nuclear RNA Auxiliary Factor 1
UBH	ubiquitin karboxy-terminální hydroláza
UBP	ubiquitin procesující hydroláza
UCHLs	ubiquitin C-terminální hydrolázy
UPS	ubiqutitin-proteazomový systém (ubiquitin-proteasome systém)
USP36	ubiquitin specifická peptidáza 36 (Ubiquitin Specific Peptidase 36)
ZAR1	Zygote Arrest Protein 1
ZP	*zona pellucida*
ZP1, ZP2,…	zona pellucida sperm-binding protein 1,2,…

8. Seznam použité literatury

Allard P, Champigny MJ, Skoggard S, Erkmann JA, Whitfield ML, Marzluff WF, Clarke HJ. 2002. Stem-loop binding protein accumulates during oocyte maturation and is not cell-cycle-regulated in the early mouse embryo. Journal of Cell Science **115**:4577–4586.

Ambros V. 2004. The functions of animal microRNAs. Nature **431**:350–355.

Aoki F, Worrad DM, Schultz RM. 1997. Regulation of Transcriptional Activity during the First and Second Cell Cycles in the Preimplantation Mouse Embryo. Developmental Biology **181**:296–307.

Bagchi S, Fredriksson R, Wallén-Mackenzie Å. 2015. In Situ Proximity Ligation Assay (PLA). Methods in Molecular Biology (Clifton, N.J.) **1318**:149–159.

Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86**:263–274.

Ball PJH, Peters AR. 2008. Reproduction in Cattle. John Wiley & Sons.

Barnes FL, Eyestone WH. 1990. Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology **33**:141–152.

Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116**:281–297.

Baumeister W, Walz J, Zühl F, Seemüller E. 1998. The proteasome: paradigm of a self-compartmentalizing protease. Cell **92**:367–380.

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmut I, Meehan R, Young L. 2004. Non-conservation of mammalian preimplantation methylation dynamics. Current biology: CB **14**:R266-267.

Bebbere D, Bogliolo L, Ariu F, Fois S, Leoni GG, Tore S, Succu S, Berlinguer F, Naitana S, Ledda S. 2008. Expression pattern of zygote arrest 1 (ZAR1), maternal antigen that embryo requires (MATER), growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) genes in ovine oocytes and in vitro-produced preimplantation embryos. Reproduction, Fertility, and Development **20**:908–915.

Bebbere D, Masala L, Albertini DF, Ledda S. 2016. The subcortical maternal complex: multiple functions for one biological structure? Journal of Assisted Reproduction and Genetics **33**:1431–1438.

Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, Pfeffer PL. 2011. Trophectoderm lineage determination in cattle. Developmental Cell **20**:244–255.

Bernstein P, Peltz SW, Ross J. 1989. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Molecular and Cellular Biology **9**:659–670.

Bianchi E, Doe B, Goulding D, Wright GJ. 2014. Juno is the egg Izumo receptor and is essential for mammalian fertilization. Nature **508**:483–487.

Blomberg L, Hashizume K, Viebahn C. 2008. Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. Reproduction (Cambridge, England) 135:181–195.

Boni R, Tosti E, Roviello S, Dale B. 1999. Intercellular communication in in vivo- and in vitro-produced bovine embryos. Biology of Reproduction **61**:1050–1055.

Borden KL. 2000. RING domains: master builders of molecular scaffolds? Journal of Molecular Biology **295**:1103–1112.

Bornstein G, Ganoth D, Hershko A. 2006. Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. Proceedings of the National Academy of Sciences of the United States of America **103**:11515–11520.

Bowman P, McLaren A. 1970. Cleavage rate of mouse embryos in vivo and in vitro. Journal of Embryology and Experimental Morphology **24**:203–207.

Boya P et al. 2005. Inhibition of macroautophagy triggers apoptosis. Molecular and Cellular Biology **25**:1025–1040.

Braude P, Bolton V, Moore S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature **332**:459–461.

Chi D, Zeng Y, Xu M, Si L, Qu X, Liu H, Li J. 2017. LC3-Dependent Autophagy in Pig 2-Cell Cloned Embryos Could Influence the Degradation of Maternal mRNA and the Regulation of Epigenetic Modification. Cellular Reprogramming **19**:354–362.

Ciechanover A, Finley D, Varshavsky A. 1984. Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85. Cell **37**:57–66.

Collart C, Allen GE, Bradshaw CR, Smith JC, Zegerman P. 2013. Titration of four replication factors is essential for the Xenopus laevis midblastula transition. Science (New York, N.Y.) **341**:893–896.

Cowling VH. 2009. Regulation of mRNA cap methylation. The Biochemical Journal **425**:295–302.

De Renzis S, Elemento O, Tavazoie S, Wieschaus EF. 2007. Unmasking activation of the zygotic genome using chromosomal deletions in the Drosophila embryo. PLoS biology **5**:e117.

Dealy MJ, Nguyen KV, Lo J, Gstaiger M, Krek W, Elson D, Arbeit J, Kipreos ET, Johnson RS. 1999. Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. Nature Genetics **23**:245–248.

Decker CJ, Parker R. 1994. Mechanisms of mRNA degradation in eukaryotes. Trends in Biochemical Sciences **19**:336–340.

DeRenzo C, Seydoux G. 2004. A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends in Cell Biology **14**:420–426.

Ducibella T, Anderson E. 1975. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. Developmental Biology **47**:45–58.

Duesbery NS, Choi T, Brown KD, Wood KW, Resau J, Fukasawa K, Cleveland DW, Vande Woude GF. 1997. CENP-E is an essential kinetochore motor in maturing oocytes and is masked during mos-dependent, cell cycle arrest at metaphase II. Proceedings of the National Academy of Sciences of the United States of America **94**:9165–9170.

Falco G, Lee S-L, Stanghellini I, Bassey UC, Hamatani T, Ko MSH. 2007. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Developmental Biology **307**:539–550.

Fang S, Weissman AM. 2004. A field guide to ubiquitylation. Cellular and molecular life sciences: CMLS **61**:1546–1561.

Filippov V, Filippova M, Sehnal F, Gill SS. 2000. Temporal and spatial expression of the cell-cycle regulator cul-1 in Drosophila and its stimulation by radiation-induced apoptosis. The Journal of Experimental Biology **203**:2747–2756.

Fraile JM et al. 2018. Loss of the deubiquitinase USP36 destabilizes the RNA helicase DHX33 and causes preimplantation lethality in mice. The Journal of Biological Chemistry **293**:2183–2194.

Fu G, Ghadam P, Sirotkin A, Khochbin S, Skoultchi AI, Clarke HJ. 2003. Mouse oocytes and early embryos express multiple histone H1 subtypes. Biology of Reproduction **68**:1569–1576.

Fu Y, Dominissini D, Rechavi G, He C. 2014. Gene expression regulation mediated through reversible m^6A RNA methylation. Nature Reviews. Genetics **15**:293–306.

Fuchs O. 2005. The role of ubiquitin-proteasome system in transforming growth factor-ß signaling and its importance in tumorigenesis. Klinická Onkologie **18**.

Galan JM, Wiederkehr A, Seol JH, Haguenauer-Tsapis R, Deshaies RJ, Riezman H, Peter M. 2001. Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. Molecular and Cellular Biology **21**:3105–3117.

Geli MI, Riezman H. 1998. Endocytic internalization in yeast and animal cells: similar and different. Journal of Cell Science **111 (Pt 8)**:1031–1037.

Gilbert SF. 2000. Early Mammalian Development. Developmental Biology. 6th edition.

Glickman MH, Ciechanover A. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiological Reviews **82**:373–428.

Glickman MH, Rubin DM, Fried VA, Finley D. 1998. The regulatory particle of the Saccharomyces cerevisiae proteasome. Molecular and Cellular Biology **18**:3149–3162.

Graf A, Krebs S, Heininen-Brown M, Zakhartchenko V, Blum H, Wolf E. 2014. Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments. Animal Reproduction Science **149**:46–58.

Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M. 2003. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. Developmental Cell **4**:799–812.

Guo H et al. 2014. The DNA methylation landscape of human early embryos. Nature **511**:606–610.

Gutierrez GJ, Vögtlin A, Castro A, Ferby I, Salvagiotto G, Ronai Z, Lorca T, Nebreda AR. 2006. Meiotic regulation of the CDK activator RINGO/Speedy by ubiquitin-proteasome-mediated processing and degradation. Nature Cell Biology **8**:1084–1094.

Hajjar C, Sampuda KM, Boyd L. 2014. Dual roles for ubiquitination in the processing of sperm organelles after fertilization. BMC developmental biology **14**:6.

Hamatani T, Carter MG, Sharov AA, Ko MSH. 2004. Dynamics of global gene expression changes during mouse preimplantation development. Developmental Cell **6**:117–131.

He C, Klionsky DJ. 2009. Regulation mechanisms and signaling pathways of autophagy. Annual Review of Genetics **43**:67–93.

Hicke L. 2001. Protein regulation by monoubiquitin. Nature Reviews. Molecular Cell Biology **2**:195–201.

Hindley CJ, McDowell GS, Wise H, Philpott A. 2011. Regulation of cell fate determination by Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligases. The International Journal of Developmental Biology **55**:249–260.

Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. 1998. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology **50**:1285–1299.

Huang DT, Hunt HW, Zhuang M, Ohi MD, Holton JM, Schulman BA. 2007. Basis for a ubiquitin-like protein thioester switch toggling E1-E2 affinity. Nature **445**:394–398.

Hwang C-S, Shemorry A, Varshavsky A. 2010. N-terminal acetylation of cellular proteins creates specific degradation signals. Science (New York, N.Y.) **327**:973–977.

Hwang K-C, Cui X-S, Park S-P, Shin M-R, Park S-Y, Kim E-Y, Kim N-H. 2004. Identification of differentially regulated genes in bovine blastocysts using an annealing control primer system. Molecular Reproduction and Development **69**:43–51.

Jia L, Bickel JS, Wu J, Morgan MA, Li H, Yang J, Yu X, Chan RC, Sun Y. 2011. RBX1 (RING box protein 1) E3 ubiquitin ligase is required for genomic integrity by modulating DNA replication licensing proteins. The Journal of Biological Chemistry **286**:3379–3386.

Jia L, Soengas MS, Sun Y. 2009. ROC1/RBX1 E3 ubiquitin ligase silencing suppresses tumor cell growth via sequential induction of G2-M arrest, apoptosis, and senescence. Cancer Research **69**:4974–4982.

Jin Y, Yang M, Gao C, Yue W, Liang X, Xie B, Zhu X, Fan S, Li R, Li M. 2019. Fbxo30 regulates chromosome segregation of oocyte meiosis. Cellular and Molecular Life Sciences **76**:2217–2229.

Jukam D, Shariati SAM, Skotheim JM. 2017. Zygotic Genome Activation in Vertebrates. Developmental Cell **42**:316–332.

Kanka J. 2003. Gene expression and chromatin structure in the pre-implantation embryo. Theriogenology **59**:3–19.

Karabinova P, Kubelka M, Susor A. 2011. Proteasomal degradation of ubiquitinated proteins in oocyte meiosis and fertilization in mammals. Cell and Tissue Research **346**:1–9.

Kelly WG. 2014. Transgenerational epigenetics in the germline cycle of Caenorhabditis elegans. Epigenetics & Chromatin **7**:6.

Kepkova KV et al. 2011. Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. Theriogenology **75**:1582–1595.

Kipreos ET, Pagano M. 2000. The F-box protein family. Genome Biology **1**:reviews3002.1-reviews3002.7.

Kline D, Kline JT. 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Developmental Biology **149**:80–89.

Latham KE, Schultz RM. 2001. Embryonic genome activation. Frontiers in Bioscience: A Journal and Virtual Library **6**:D748-759.

Laurincík J, Hyttel P, Baran V, Eckert J, Lucas-Hahn A, Pivko J, Niemann H, Brem G, Schellander K. 1998. A detailed analysis of pronucleus development in bovine zygotes in vitro: cell-cycle chronology and ultrastructure. Molecular Reproduction and Development **50**:192–199.

Lee HR, Kim DH, Kim MG, Lee JS, Hwang JH, Lee HT. 2016. The regulation of autophagy in porcine blastocysts: Regulation of PARylation-mediated autophagy via mammalian target of rapamycin complex 1 (mTORC1) signaling. Biochemical and Biophysical Research Communications **473**:899–906.

Lee KY, DeMayo FJ. 2004. Animal models of implantation. Reproduction (Cambridge, England) **128**:679–695.

Lee MT, Bonneau AR, Giraldez AJ. 2014. Zygotic genome activation during the maternal-to-zygotic transition. Annual review of cell and developmental biology **30**:581–613.

Lee S-E, Hwang K-C, Sun S-C, Xu Y-N, Kim N-H. 2011. Modulation of autophagy influences development and apoptosis in mouse embryos developing in vitro. Molecular Reproduction and Development **78**:498–509.

Lee YL, Lee KF, Xu JS, Kwok KL, Luk JM, Lee WM, Yeung WSB. 2003. Embryotrophic factor-3 from human oviductal cells affects the messenger RNA expression of mouse blastocyst. Biology of Reproduction **68**:375–382.

Lefièvre L et al. 2004. Four zona pellucida glycoproteins are expressed in the human. Human Reproduction (Oxford, England) **19**:1580–1586.

Li L, Lu X, Dean J. 2013. The maternal to zygotic transition in mammals. Molecular Aspects of Medicine **34**:919–938.

Li X-Y, Harrison MM, Villalta JE, Kaplan T, Eisen MB. 2014. Establishment of regions of genomic activity during the Drosophila maternal to zygotic transition. eLife **3**.

Lipford JR, Smith GT, Chi Y, Deshaies RJ. 2005. A putative stimulatory role for activator turnover in gene expression. Nature **438**:113–116.

Liu D et al. 2006. Interaction of Skp1 with CENP-E at the midbody is essential for cytokinesis. Biochemical and Biophysical Research Communications **345**:394–402.

Liu J, Furukawa M, Matsumoto T, Xiong Y. 2002. NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. Molecular Cell **10**:1511–1518.

Livneh I, Cohen-Kaplan V, Cohen-Rosenzweig C, Avni N, Ciechanover A. 2016. The life cycle of the 26S proteasome: from birth, through regulation and function, and onto its death. Cell Research **26**:869–885.

Lu X, Gao Z, Qin D, Li L. 2017. A Maternal Functional Module in the Mammalian Oocyte-To-Embryo Transition. Trends in Molecular Medicine **23**:1014–1023.

Maddox-Hyttel P, Svarcova O, Laurincik J. 2007. Ribosomal RNA and nucleolar proteins from the oocyte are to some degree used for embryonic nucleolar formation in cattle and pig. Theriogenology **68 Suppl 1**:S63-70.

Mandel SA, Fishman-Jacob T, Youdim MBH. 2012. Targeting SKP1, an ubiquitin E3 ligase component found decreased in sporadic Parkinson's disease. Neuro-Degenerative Diseases **10**:220–223.

Marangos P, Verschuren EW, Chen R, Jackson PK, Carroll J. 2007. Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1). The Journal of Cell Biology **176**:65–75.

Mayer W, Niveleau A, Walter J, Fundele R, Haaf T. 2000. Demethylation of the zygotic paternal genome. Nature **403**:501–502.

McLay DW, Clarke HJ. 2003. Remodelling the paternal chromatin at fertilization in mammals. Reproduction (Cambridge, England) **125**:625–633.

Meiners S, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel P-M, Krüger E. 2003. Inhibition of proteasome activity induces concerted expression of proteasome genes and de novo formation of Mammalian proteasomes. The Journal of Biological Chemistry **278**:21517–21525.

Mellman I. 1996. Endocytosis and molecular sorting. Annual Review of Cell and Developmental Biology **12**:575–625.

Minami N, Suzuki T, Tsukamoto S. 2007. Zygotic gene activation and maternal factors in mammals. The Journal of Reproduction and Development **53**:707–715.

Moore R, Boyd L. 2004. Analysis of RING finger genes required for embryogenesis in C. elegans. Genesis (New York, N.Y.: 2000) **38**:1–12.

Morimoto M, Nishida T, Nagayama Y, Yasuda H. 2003. Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. Biochemical and Biophysical Research Communications **301**:392–398.

Mtango NR, Latham KE. 2007. Ubiquitin proteasome pathway gene expression varies in rhesus monkey oocytes and embryos of different developmental potential. Physiological Genomics **31**:1–14.

Mtango NR, Latham KE, Sutovsky P. 2014. Deubiquitinating enzymes in oocyte maturation, fertilization and preimplantation embryo development. Advances in Experimental Medicine and Biology **759**:89–110.

Mtango NR, Sutovsky M, Susor A, Zhong Z, Latham KE, Sutovsky P. 2012. Essential role of maternal UCHL1 and UCHL3 in fertilization and preimplantation embryo development. Journal of Cellular Physiology **227**:1592–1603.

Mújica A, Navarro-García F, Hernández-González EO, De Lourdes Juárez-Mosqueda M. 2003. Perinuclear theca during spermatozoa maturation leading to fertilization. Microscopy Research and Technique **61**:76–87.

Ohsugi M, Zheng P, Baibakov B, Li L, Dean J. 2008. Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. Development (Cambridge, England) **135**:259–269.

Ohta T, Michel JJ, Xiong Y. 1999. Association with cullin partners protects ROC proteins from proteasome-dependent degradation. Oncogene **18**:6758–6766.

Osley MA. 2004. H2B ubiquitylation: the end is in sight. Biochimica Et Biophysica Acta **1677**:74–78.

Pan H, O'brien MJ, Wigglesworth K, Eppig JJ, Schultz RM. 2005. Transcript profiling during mouse oocyte development and the effect of gonadotropin priming and development in vitro. Developmental Biology **286**:493–506.

Pan Z-Q, Kentsis A, Dias DC, Yamoah K, Wu K. 2004. Nedd8 on cullin: building an expressway to protein destruction. Oncogene **23**:1985–1997.

Pangas SA, Li X, Robertson EJ, Matzuk MM. 2006. Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. Molecular Endocrinology (Baltimore, Md.) **20**:1406–1422.

Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbiès-Tran R. 2004. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. Biology of Reproduction **71**:1359–1366.

Petroski MD, Deshaies RJ. 2005. Function and regulation of cullin-RING ubiquitin ligases. Nature Reviews. Molecular Cell Biology **6**:9–20.

Pickart CM. 2001. Mechanisms underlying ubiquitination. Annual Review of Biochemistry **70**:503–533.

Piva R, Liu J, Chiarle R, Podda A, Pagano M, Inghirami G. 2002. In vivo interference with Skp1 function leads to genetic instability and neoplastic transformation. Molecular and Cellular Biology **22**:8375–8387.

Rhodes SL, Fitzmaurice AG, Cockburn M, Bronstein JM, Sinsheimer JS, Ritz B. 2013. Pesticides that inhibit the ubiquitin-proteasome system: effect measure modification by genetic variation in SKP1 in Parkinson′s disease. Environmental Research **126**:1–8.

Rojansky R, Cha M-Y, Chan DC. 2016. Elimination of paternal mitochondria in mouse embryos occurs through autophagic degradation dependent on PARKIN and MUL1. eLife **5**.

Salilew-Wondim D, Hölker M, Rings F, Phatsara C, Mohammadi-Sangcheshmeh A, Tholen E, Schellander K, Tesfaye D. 2010. Depletion of BIRC6 leads to retarded bovine early embryonic development and blastocyst formation in vitro. Reproduction, Fertility, and Development **22**:564–579.

Sasagawa Y, Urano T, Kohara Y, Takahashi H, Higashitani A. 2003. Caenorhabditis elegans RBX1 is essential for meiosis, mitotic chromosomal condensation and segregation, and cytokinesis. Genes to Cells: Devoted to Molecular & Cellular Mechanisms **8**:857–872.

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. 2002. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development (Cambridge, England) **129**:3533–3544.

Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell **75**:495–505.

Schultz RM. 2005. From egg to embryo: a peripatetic journey. Reproduction (Cambridge, England) **130**:825–828.

Schulz KN, Harrison MM. 2018. Mechanisms regulating zygotic genome activation. Nature Reviews. Genetics.

Shimizu K, Nihira NT, Inuzuka H, Wei W. 2018. Physiological functions of FBW7 in cancer and metabolism. Cellular Signalling **46**:15–22.

Shimizu N et al. 2014. Possible role of ZPAC, zygote-specific proteasome assembly chaperone, during spermatogenesis in the mouse. The Journal of Reproduction and Development **60**:179–186.

Shimuta K, Nakajo N, Uto K, Hayano Y, Okazaki K, Sagata N. 2002. Chk1 is activated transiently and targets Cdc25A for degradation at the Xenopus midblastula transition. The EMBO journal **21**:3694–3703.

Shin S-W et al. 2010. Inhibition of the ubiquitin-proteasome system leads to delay of the onset of ZGA gene expression. The Journal of Reproduction and Development **56**:655–663.

Shin S-W et al. 2013. Mouse zygote-specific proteasome assembly chaperone important for maternal-to-zygotic transition. Biology Open **2**:170–182.

Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW. 1997. F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91**:209–219.

Song B-S et al. 2012. Induction of autophagy promotes preattachment development of bovine embryos by reducing endoplasmic reticulum stress. Biology of Reproduction **87**:8, 1–11.

Su Y-Q, Sugiura K, Woo Y, Wigglesworth K, Kamdar S, Affourtit J, Eppig JJ. 2007. Selective degradation of transcripts during meiotic maturation of mouse oocytes. Developmental Biology **302**:104–117.

Sun L, Bertke MM, Champion MM, Zhu G, Huber PW, Dovichi NJ. 2014. Quantitative proteomics of Xenopus laevis embryos: expression kinetics of nearly 4000 proteins during early development. Scientific Reports **4**:4365.

Suresh B, Lee J, Kim K-S, Ramakrishna S. 2016. The Importance of Ubiquitination and Deubiquitination in Cellular Reprogramming. Stem Cells International **2016**.

Susor A et al. 2015. Temporal and spatial regulation of translation in the mammalian oocyte via the mTOR-eIF4F pathway. Nature Communications **6**:6078.

Susor A, Liskova L, Toralova T, Pavlok A, Pivonkova K, Karabinova P, Lopatarova M, Sutovsky P, Kubelka M. 2010. Role of ubiquitin C-terminal hydrolase-L1 in antipolyspermy defense of mammalian oocytes. Biology of Reproduction **82**:1151–1161.

Sutovsky P et al. 2001. Accumulation of the proteolytic marker peptide ubiquitin in the trophoblast of mammalian blastocysts. Cloning and Stem Cells **3**:157–161.

Svoboda P. 2018. Mammalian zygotic genome activation. Seminars in Cell & Developmental Biology **84**:118–126.

Tan JMM et al. 2008. Lysine 63-linked ubiquitination promotes the formation and autophagic clearance of protein inclusions associated with neurodegenerative diseases. Human Molecular Genetics **17**:431–439.

Tan M, Davis SW, Saunders TL, Zhu Y, Sun Y. 2009. RBX1/ROC1 disruption results in early embryonic lethality due to proliferation failure, partially rescued by simultaneous loss

of p27. Proceedings of the National Academy of Sciences of the United States of America **106**:6203–6208.

Tay TL, Lin Q, Seow TK, Tan KH, Hew CL, Gong Z. 2006. Proteomic analysis of protein profiles during early development of the zebrafish, Danio rerio. Proteomics **6**:3176–3188.

Telford NA, Watson AJ, Schultz GA. 1990. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Molecular Reproduction and Development **26**:90–100.

Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. 2000. Recognition of the polyubiquitin proteolytic signal. The EMBO journal **19**:94–102.

Tong S, Si Y, Yu H, Zhang L, Xie P, Jiang W. 2017. MLN4924 (Pevonedistat), a protein neddylation inhibitor, suppresses proliferation and migration of human clear cell renal cell carcinoma. Scientific Reports **7**:5599.

Topper EK, Kruijt L, Calvete J, Mann K, Töpfer-Petersen E, Woelders H. 1997. Identification of bovine zona pellucida glycoproteins. Molecular Reproduction and Development **46**:344–350.

Toralová T, Benešová V, Kepková KV, Vodička P, Šušor A, Kaňka J. 2012. Bovine preimplantation embryos with silenced nucleophosmin mRNA are able to develop until the blastocyst stage. Reproduction (Cambridge, England) **144**:349–359.

Toralová T, Susor A, Nemcová L, Kepková K, Kanka J. 2009. Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage. Reproduction (Cambridge, England) **138**:783–791.

Tripurani SK, Wee G, Lee K-B, Smith GW, Wang L, JianboYao. 2013. MicroRNA-212 Post-Transcriptionally Regulates Oocyte-Specific Basic-Helix-Loop-Helix Transcription Factor, Factor in the Germline Alpha (FIGLA), during Bovine Early Embryogenesis. PLOS ONE **8**:e76114.

Tsukamoto S, Hara T, Yamamoto A, Kito S, Minami N, Kubota T, Sato K, Kokubo T. 2014. Fluorescence-based visualization of autophagic activity predicts mouse embryo viability. Scientific Reports **4**:4533.

Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. 2008. Autophagy is essential for preimplantation development of mouse embryos. Science (New York, N.Y.) **321**:117–120.

Tsukamoto S, Tatsumi T. 2018. Degradation of maternal factors during preimplantation embryonic development. The Journal of Reproduction and Development **64**:217–222.

Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H. 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Current biology: CB **9**:661–664.

Valadão L, Silva HM da, Silva FM da. 2018. Bovine Embryonic Development to Implantation. Embryology - Theory and Practice.

Van Soom A, Boerjan ML, Bols PE, Vanroose G, Lein A, Coryn M, de Kruif A. 1997. Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. Biology of Reproduction **57**:1041–1049.

Vanroose G, Nauwynck H, Soom AV, Ysebaert M-T, Charlier G, Oostveldt PV, de Kruif A. 2000. Structural Aspects of the Zona Pellucida of In Vitro-Produced Bovine Embryos: A Scanning Electron and Confocal Laser Scanning Microscopic Study. Biology of Reproduction **62**:463–469.

Varshavsky A. 1991. Naming a targeting signal. Cell **64**:13–15.

Verlhac M-H, Terret M-E, Pintard L. 2010. Control of the oocyte-to-embryo transition by the ubiquitin-proteolytic system in mouse and C. elegans. Current Opinion in Cell Biology **22**:758–763.

Vigneault C, Gravel C, Vallée M, McGraw S, Sirard M-A. 2009. Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. Reproduction (Cambridge, England) **137**:245–257.

Wahle E, Winkler GS. 2013. RNA decay machines: deadenylation by the Ccr4-not and Pan2-Pan3 complexes. Biochimica Et Biophysica Acta **1829**:561–570.

Walser CB, Lipshitz HD. 2011. Transcript clearance during the maternal-to-zygotic transition. Current Opinion in Genetics & Development **21**:431–443.

Wamaitha SE, Niakan KK. 2018. Human Pre-gastrulation Development. Current Topics in Developmental Biology **128**:295–338.

Wan M, Tang Y, Tytler EM, Lu C, Jin B, Vickers SM, Yang L, Shi X, Cao X. 2004. Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. The Journal of Biological Chemistry **279**:14484–14487.

Wang S, Kou Z, Jing Z, Zhang Y, Guo X, Dong M, Wilmut I, Gao S. 2010. Proteome of mouse oocytes at different developmental stages. Proceedings of the National Academy of Sciences of the United States of America **107**:17639–17644.

Wang Y, Penfold S, Tang X, Hattori N, Riley P, Harper JW, Cross JC, Tyers M. 1999. Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. Current biology: CB **9**:1191–1194.

Watson AJ, Barcroft LC. 2001. Regulation of blastocyst formation. Frontiers in Bioscience: A Journal and Virtual Library **6**:D708-730.

Weill L, Belloc E, Bava F-A, Méndez R. 2012. Translational control by changes in poly(A) tail length: recycling mRNAs. Nature Structural & Molecular Biology **19**:577–585.

Weissman AM. 2001. Themes and variations on ubiquitylation. Nature Reviews. Molecular Cell Biology **2**:169–178.

Wirbelauer C, Sutterlüty H, Blondel M, Gstaiger M, Peter M, Reymond F, Krek W. 2000. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. The EMBO journal **19**:5362–5375.

Wójcik C, DeMartino GN. 2003. Intracellular localization of proteasomes. The International Journal of Biochemistry & Cell Biology **35**:579–589.

Xiao H, Wang H, Silva EA, Thompson J, Guillou A, Yates JR, Buchon N, Franc NC. 2015. The Pallbearer E3 ligase promotes actin remodeling via RAC in efferocytosis by degrading the ribosomal protein S6. Developmental Cell **32**:19–30.

Yan L et al. 2013. Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nature Structural & Molecular Biology **20**:1131–1139.

Yang Y et al. 2017. The E3 ubiquitin ligase RNF114 and TAB1 degradation are required for maternal-to-zygotic transition. EMBO reports **18**:205–216.

Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y. 1998. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. Nature **396**:590–594.

Yartseva V, Giraldez AJ. 2015. The Maternal-to-Zygotic Transition During Vertebrate Development: A Model for Reprogramming. Current Topics in Developmental Biology **113**:191–232.

Ye X, Nalepa G, Welcker M, Kessler BM, Spooner E, Qin J, Elledge SJ, Clurman BE, Harper JW. 2004. Recognition of phosphodegron motifs in human cyclin E by the SCF(Fbw7) ubiquitin ligase. The Journal of Biological Chemistry **279**:50110–50119.

Yi Y-J, Nagyova E, Manandhar G, Procházka R, Sutovsky M, Park C-S, Sutovsky P. 2008. Proteolytic activity of the 26S proteasome is required for the meiotic resumption, germinal vesicle breakdown, and cumulus expansion of porcine cumulus-oocyte complexes matured in vitro. Biology of Reproduction **78**:115–126.

Yokoi H, Natsuyama S, Iwai M, Noda Y, Mori T, Mori K, Fujita K, Nakayama H, Fujita J. 1993. Nonradioisotopic Quantitative Rt-Pcr to Detect Changes in Messenger-Rna

Levels During Early Mouse Embryo Development. Biochemical and Biophysical Research Communications **195**:769–775.

Zhang H, Kobayashi R, Galaktionov K, Beach D. 1995. p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase. Cell **82**:915–925.

Zhang J, Tomasini AJ, Mayer AN. 2008a. RBM19 is essential for preimplantation development in the mouse. BMC developmental biology **8**:115.

Zhang M, Botër M, Li K, Kadota Y, Panaretou B, Prodromou C, Shirasu K, Pearl LH. 2008b. Structural and functional coupling of Hsp90- and Sgt1-centred multi-protein complexes. The EMBO Journal **27**:2789–2798.

Zhang P, Zucchelli M, Bruce S, Hambiliki F, Stavreus-Evers A, Levkov L, Skottman H, Kerkelä E, Kere J, Hovatta O. 2009. Transcriptome Profiling of Human Pre-Implantation Development. PLOS ONE **4**:e7844.

Zhang Q et al. 2015. The novel protective role of P27 in MLN4924-treated gastric cancer cells. Cell Death & Disease **6**:e1867.

Zhang Q, Chen Q, Lu X, Zhou Z, Zhang H, Lin H-Y, Duan E, Zhu C, Tan Y, Wang H. 2013. CUL1 promotes trophoblast cell invasion at the maternal-fetal interface. Cell Death & Disease **4**:e502.

Zheng J, Yang X, Harrell JM, Ryzhikov S, Shim EH, Lykke-Andersen K, Wei N, Sun H, Kobayashi R, Zhang H. 2002a. CAND1 binds to unneddylated CUL1 and regulates the formation of SCF ubiquitin E3 ligase complex. Molecular Cell **10**:1519–1526.

Zheng N et al. 2002b. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature **416**:703–709.

Zhou L et al. 2013. The role of RING box protein 1 in mouse oocyte meiotic maturation. PloS One **8**:e68964.

Zhou L, Zhang W, Sun Y, Jia L. 2018. Protein neddylation and its alterations in human cancers for targeted therapy. Cellular signalling **44**:92–102.

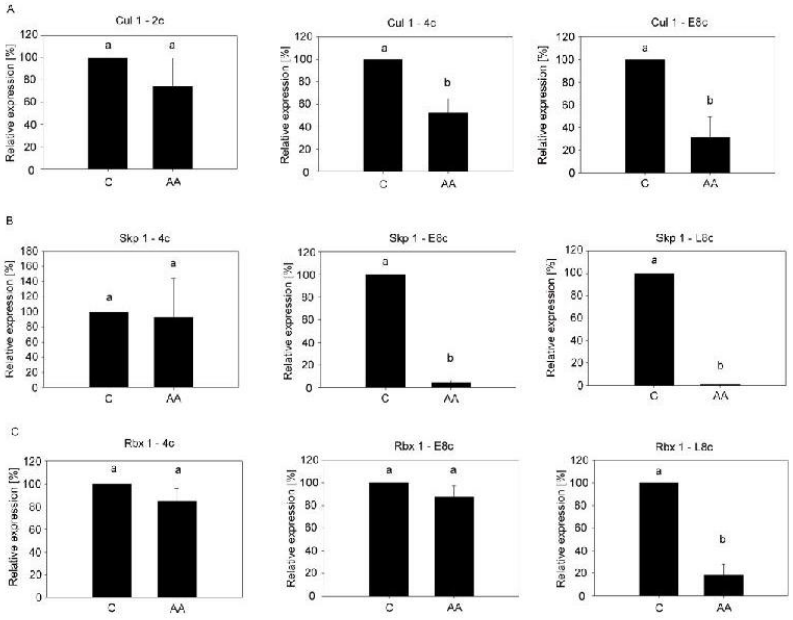
9. Seznam obrázků

10. Přílohy

10.1. Příloha k článku Charakterizace SCF komplexu v průběhu preimplantačního vývoje skotu

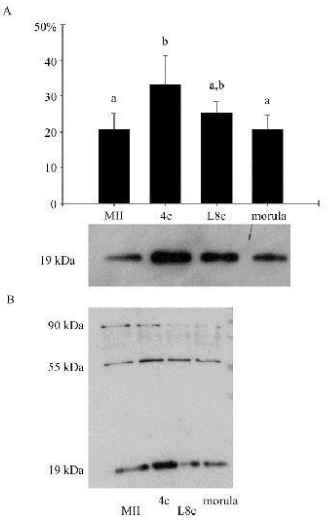
Supporting Information

S1 Fig. Relative mRNA expression of invariant members of SCF complex, embryos treated with α-amanitin, supplement. The data were normalised according to the relative concentration of the external standard (luciferase mRNA, 1pg per embryo). (A) *Cul1*, (B) *Skp1*, (C) *Rbx1*. Bars show ± S.D. [a,b] Values with different superscripts indicate statistical significance (P<0.05). (C, control group of untreated embryos; AA, group of embryos treated with α-amanitin; 2c, two-cell stage embryo; 4c, four-cell stage embryo; E8c, early eight-stage embryo; L8c, late eight-cell stage embryo).

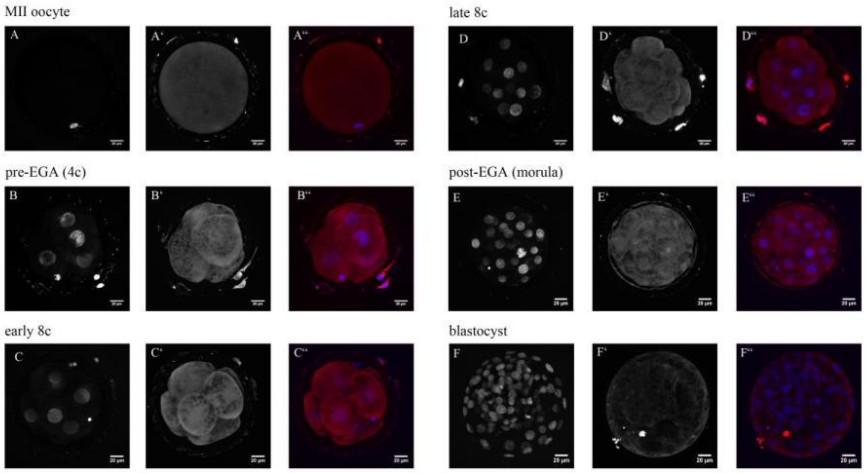


S2 Fig. Western blot analysis of bovine oocytes and preimplantation embryos using anti-SKP1 antibody 1H9. 30 embryos per lane. A) Quantification of protein level. The data were

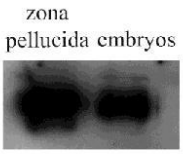
processed using Quantity One software (Bio-Rad). 100% represents the sum of the trace quantities of all bands; relative abundance (y-axis) represents the percentage of each band. Bars show mean ± S.D. [a,b]Values with different superscripts indicate statistical significance ($P<0.05$). The experiment was repeated four times, and a representative western blot image is shown below the graph. B) Representative image of additional bands (approximate size 55 and 90 kDa). (MII, MII oocytes; L8c, late eight-cell-stage embryos; 4c, four-cell-stage embryos).



S3 Fig. Confocal laser scanning microscopy of SKP1 (antibody 1H9) from MII oocytes to blastocyst-stage embryos. The embryos were labelled with mouse monoclonal anti-SKP1 antibody 1H9 (A' - F') and the nuclei were stained with DAPI (A - F). In overlaid images (A'' – F''), SKP1 is red and DNA blue.



S4 Fig. Western blot analysis of SKP1 using Abnova antibody. First band shows SKP1 on zona pellucida, second band shows SKP1 in embryos.



10.2. Příloha k článku Inhibice Skp1-Cullin-F-box komplexů v průběhu zrání oocytů a preimplantačního vývoje skotu vede k opožděnému vývoji embryí

Supplementary material

Material and Methods

MLN4924 treatment – the optimal dose determination

The cultivation with MLN4924 inhibitor was performed in the same way as is described in the main text. MLN4924 was added to the culture medium at several concentration to determine the optimal dose of this inhibitor. Used concentrations were: 0.3 μM, 1 μM, 1,5 μM and 3 μM. Embryos were treated from 4c to L8c stage (44 – 96 hpf), subsequently washed and cultivated in Menezo B2 medium until the blastocyst stage.

Synthesis of Cul1 DNA template and dsRNA

The RNA for Cul1 DNA template synthesis was isolated from bovine fibroblasts or oocytes using RNeasy Mini Kit (Qiagen). The template was synthesized using primers with T7 promotors:

Cul1 dsRNA

F: 5′-AGGATCCGCTAGCTAATACGACTCACTATAGGGAGATGGAGACACACATTC

ATAATCAGGG-3′

R: 5′- ACTCGAGGCTAGCTAATACGACTCACTATAGGGAGACACCAATGTCTTGAA

ACATCCGCT-3′

GFP dsRNA

F: 5′-AGGATCCTAATACGACTAACTATAGGGAGAATGGTGAGCAAGGGCGAGGA-3′
R: 5′-ACTCGAGTAATACGACTCACTATAGGGAGAGCGGCCGCTTTACTTGTACA-3′
The RT was performed at 60 °C using two-step Phusion RT-PCR kit (Finnzymes, Vantaa, Finland) primed with Oligo (dT) primers. The DNA template coupled with T7 promotor was in vitro transcribed using MEGAscript RNAi Kit (ThermoFisher Scientific, Prague, Czech Republic). An amount of 1.2 μg of DNA template was used for each reaction. The reaction mixture was incubated for 4 h at 37 °C and the sense and antisense strands were transcribed in the same reaction. The residual DNA template and ssRNA were digested and the dsRNA was purified according to the manufacturer's instruction. One microliter RNA acquired by in vitro

transcription and 1 µl of final dsRNA were resolved by electrophoresis on 1.5 % agarose gel to confirm the integrity of the dsRNA and efficiency of the annealing step.

Zygote microinjection

The zygotes were injected 20 hpf at the stage of two pronuclei. dsRNA was dissolved in RNase-free water to a final concentration of 800 ng/µl. Two control group were establish – the uninjected control and a group injected with GFP dsRNA.

Zygotes were injected with ~ 5pl of the dsRNA using an inverted microscope Leica DMI 6000B with Transferman NK2 and Femtojet (Eppendorf Czech & Slovakia s.r.o., Ricany u Prahy, Czech Republic). Pipettes for microinjection were made using P97 Pipette Puller (Sutter Instrument Company, Novato, CA, USA). In total, 75 embryos were included in the study three independent injection sessions. Embryos were categorized into the following groups: 1) embryos injected with Cul1 dsRNA (30 embryos), 2) embryos injected with GFP dsRNA (30) and 3) uninjected embryos (15). After microinjection, embryos were cultivated under the conditions mentioned in Material and Methods in the main text and collected at specific developmental stages.

Western blot

The western blot analysis was performed as described in the main text. The antibodies used were as follows: rabbit anti-Cullin 1 (ab75812) and rabbit anti-Cullin 1 (ab199415) both 1:1000 in 5% non-fat milk (Abcam, Cambridge, UK).

Results

Determination of the optimal dose of MLN4924

To determine the optimal concentration of MLN4924, several concentration of MLN4924 (0.3 µM, 1 µM, 1.5 µM and 3 µM) were used. The development of embryos treated with 0.3 µM from the 4c to late 8c stage was not significantly changed ($p>0.05$; S3A Fig.). The rates of development to the blastocyst stage after treatment with 1.5 µM MLN4924 were significantly lower compared to control (1.5 µM MLN: 2.083 ± 3.608 %, $p= 0.005$; mean ± S.D., the number of 4-cell stage embryos was considered to be 100 %; control: 27.38 ± 2.061%, S3B Fig.). When 3 µM MLN4924 was used, only 9.84 ± 10.004 % ($p=0,015$) were able to reach the morula stage compared to 54.167 ± 16.009 % control embryos and these embryos never developed into blastocyst (S3C Fig.). Moreover, part of treated embryos shows morphological changes, under

the stereomicroscopy they look like homogeneous ball of cytoplasm and never developed into the subsequent stage. Cultivation with 1 μM MLN4924 showed decreasing numbers of developing embryos from morula to blastocyst stages (168 hpf), but some of the embryos were able to reach the blastocyst stage later (192 hpf, Fig. 1 in the main text). That showed us the ability of embryos to overcome this handicap and restore the activity of SCF complex. For these reasons, the treatment with 1μM of MLN4924 from 4cell to late 8cell stage was considered to be the lowest functional concentration.

Verification of Cullin 1 mRNA and protein silencing

To prevent the formation of SCF complex, zygotes were microinjected with specific dsRNA against Cullin 1 mRNA. The microinjection of cullin 1 dsRNA efficiently (S1 Fig.) caused degradation of cullin 1 mRNA in bovine preimplantation embryos. The Cullin 1 mRNA was reduced by 74.69 % in comparison to uninjected control ($p<0.001$) and by 72.31 % ($p=0.004$) in comparison to GFP dsRNA injected control. No significant difference was found in the abundance of cullin 1 mRNA between the uninjected group and GFP dsRNA-injected group ($p>0.05$). The experiment was repeated three times.

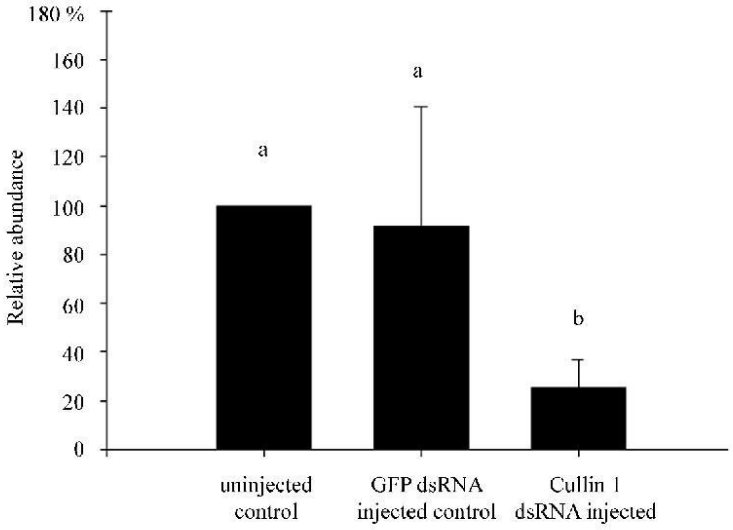
The level of CUL1 protein in microinjected embryos was analyzed using western blot. No significant decrease in CUL1 protein in Cul1 dsRNA injected embryos was found. The experiment was repeated three times using two different antibodies (S2 Fig.).

Tables

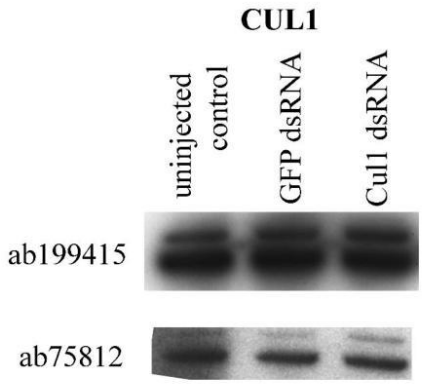
Antibody	Vendor	Cat. number	Host Organism	Dilution
Anti-CENPE	Cell Signaling Technology	14977	rabbit	1:500
Anti-IKBA	Cell Signaling Technology	9242S	rabbit	1:1000
Anti-P27	Abcam	Ab32034	rabbit	1:1000
Anti-RBM19	Abcam	Ab122515	rabbit	1:250
Anti-RPS6	Santa Cruz Biotechnology	SC-74459	mouse	1:1000
Anti-SMAD4	Proteintech	10231-I-AP	rabbit	1: 7000
Anti-ZAR1	Bioss Antibodies	bs-13549R	rabbit	1:300
Anti-alpha-Tubulin	Abcam	ab52866	rabbit	1:2000
Anti-Lamin	Sigma-Aldrich	SAB4200236	mouse	1:150

Supplementary table 1. Antibody Table

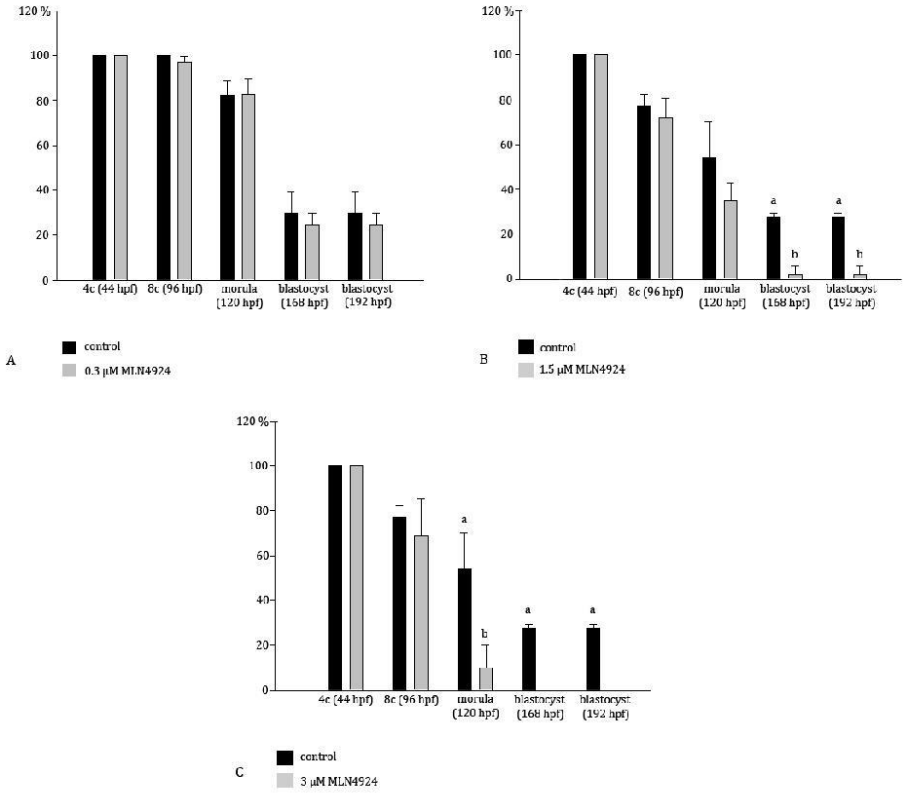
Figures



S1 Figure


CUL1
uninjected control
GFP dsRNA
Cul1 dsRNA
ab199415
ab75812

S2 Figure


120 %
100
80
60
40
20
0
4c (44 hpf)
8c (96 hpf)
morula (120 hpf)
blastocyst (168 hpf)
blastocyst (192 hpf)
control
0.3 µM MLN4924
A
a
b
1.5 µM MLN4924
B
3 µM MLN4924
C

S3 Figure